SUSTAINABILITY OF ACTIVATED SLUDGE PROCESS FOR SIMULTANEOUS NUTRIENTS REMOVAL AND SLUDGE MINIMIZATION

by

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ABSTRACT

Sludge reduction at source for the sludge minimization through fasting and feasting has been practiced with activated sludge processes over the past few decades. In this research, two sequencing batch reactors (SBRs) were operated aiming to investigate the possibility of simultaneous sludge reduction and nutrient removal using both synthetic and real wastewater. One of the lab-scale reactors (called the control-SBR) was run in a standard operational mode at 10-day solid retention time (SRT), while the other reactor (called the modified-SBR) was run in a sludge minimizing mode to induce the anaerobiosis of the returned biomass in a sidestream reactor. Furthermore, to compare the overall biomass yields in both reactors, the waste biomass from the control-SBR was taken to a conventional anaerobic digester. Both SBRs were fed with synthetic wastewater, and then changed to real primary effluent in a step-wise manner from one municipal wastewater treatment plant (WWTP), and then it was fed with the raw wastewater (after being screened) from another WWTP.

Overall, both reactors achieved a higher than 80% of PO_4^{3-}-P removal and 95% of NH_4^+-N removal. The modified system generated 60% less biomass than the control system with synthetic wastewater. The sludge reduction achieved in the modified system was 39% and 35%, compared to the control system, when the reactors were fed with real primary effluent and raw wastewater, respectively. Carbon mass balance and partitioning experiments showed that the modified-SBR had better mineralization in terms of CO_2

production. In the modified-SBR, less ^{13}C partitioned into biomass and more ^{13}C went into the headspace in the form of CO_2, thus suggesting why modified-SBR achieved a lower biomass yield.

Furthermore, modified-SBR contained more diverse ammonia oxidizing bacteria and polyphosphate accumulating bacteria (PAOs) than in the control-SBR. Since it contained more slow growing bacteria (*Nitrospira*, *Mesorhizobium* and *Candidatus* Accumulibacter) and filamentous bacteria (unclassified *Cytophagales*), this could be another possible mechanism of sludge reduction in the modified-SBR. Two *Dechloromonas*-related operational taxonomic unites (OTUs) were detected in both SBRs, as the denitrifying PAOs that could utilize nitrite or nitrate to remove phosphorus without any extracellular carbon substrates under anoxic conditions.

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INTRODUCTION

Activated Sludge Process

The activated sludge process (ASP) has become widespread throughout the world, since 1930 (Benidickson and Jamie, 2011). This process deals with municipal and industrial sewage (Grady et al., 1999; Metcalf and Eddy, 1994). The process can be optimized for biological removal of nitrogen and phosphorus using different reactor configurations in addition to the effective removal of organic matter and suspended solids to comply with the effluent limitations and monitoring requirements. These include the national pollutant elimination discharge system (NPEDS) and the Total Mass Discharge Limits (TMDL) (Grady et al., 1999; Metcalf and Eddy, 1994). Figure 1.1 shows a typical configuration of an activated sludge process bioreactor. The primary effluent (i.e., influent to the bioreactor) is fed to the ASP bioreactor along with the recycled flow (returned biomass). A consortium of microorganisms acts upon organic carbon, nitrogen and phosphorus, when the wastewater containing these contaminants passes through various sequential oxygen rich (aerobic) and oxygen free (anoxic and anaerobic) zones. As a result, these contaminants are either oxidized to their gaseous forms or converted to their less harmful soluble forms. Consequentially, microorganisms gain energy from these metabolic reactions and grow. The treated liquid waste flows by gravity to the secondary clarifier where most of the biomass settles to the bottom of the clarifier. Because wastewater treatment is a continuous process, the settled biomass in the

secondary clarifier is routinely removed from the bottom. A small portion of this removed biomass is recycled back into the bioreactor in order to maintain a sustainable population of bacteria in the bioreactor, and a larger portion is turned into waste on daily basis.

This waste active sludge (WAS) is the unpleasant byproduct of ASP. It is a large volume, and it is expensive to treat. Currently, approximately 8.2 million tons of WAS is generated per year in the United States, and the European Union annually produces over 10 million tons (USEPA, 1999, Wang et al., 2012, Wang et al., 2013). Moreover, the WAS produced annually will continue to increase in future (Guo et al., 2010).

The treatment of the excess sludge is labor, energy, and dollar intensive; it may consume as much as 65% of the plant's operations expenses (Saby et. al., 2003; Chen et al., 2001 and 2003; Camacho et al., 2002; Cui and Jahng, 2004; Barjenbruch and Kopplow, 2003). One option for the use of sludge includes its composting followed by land application. However, land application of sludge is restricted in many states due to due to health risks to people and livestock. There is a potential for secondary pollution by the emission of methane or greenhouse gas. There can also be toxic elements in the sewage sludge, i.e., heavy metals, pathogens, pharmaceuticals, and nutrients (Wei et al., 2003; Kim et al., 2012). The handling and disposal of excess sludge is more challenging in coastal areas such as Florida and California, and in coastal countries like Malaysia, Singapore and Indonesia, due to the depleting of landfill resources and other environmental concerns. With increasing urbanization and industrialization the sludge problem will be increased and become more challenging. Incineration decreases the volume of solids by up to 95%. However, it requires expensive machinery, consumes

nonrenewable resources, and has a negative public impression (Tchobanoglus et al., 2003). Sludge reduction at the wastewater treatment plants becomes a more acceptable alternative solution to solve sludge-associated problems.

Sludge Reduction Processes

There are two categories of technologies or strategies that have been developed to minimize the waste sludge (Mahmood and Elliott, 2006): 1) sludge reduction through posttreatment; 2) sludge reduction at the source. Anaerobic and aerobic digestion are the most common methods of posttreatment sludge and it can reduce the excess biomass by 40~50%. However, they are capital intensive, process-wise complex and chemical dosing is required as well (Khursheed and Kazmi, 2011). Therefore, sludge reduction at the source is generally preferred over posttreatment sludge, as it contributes to a cascading decrease in sludge handling, stabilization, transportation, and disposal expenses.

For sludge reduction at the source, a number of technologies have been developed. Figure 1.2 includes lysis-cryptic methods combined with the activated sludge processes (He and Wei, 2010), sludge reduction based on uncoupling metabolism (Feng et al., 2012) and worms' predation (Lou et al., 2011). Böhler and Siegrist (2006) claimed that all other physical, chemical, biological, and thermal processes are expensive and could increase the overall energy consumption of a plant. Guo et al. (2013) after reviewing all of the technologies listed above, concluded that sludge reduction through fasting and feasting (Westgarth, 1963; Novak et al., 2007; Chen et al., 2001) had an obvious more positive effect compared to the others, because 1) there is no extra chemical or physical addition required, 2) it improves the sedimentation, 3) it is capable of treating complex components or high strength organic pollutants, 4) it is flexible to

operate and easy to be meliorated, and 5) it is economically efficient and environmentally friendly.

Sludge reduction through the fasting and feasting process has been primarily investigated in laboratory scale setups with few full-scale installations in the U.S. These have the trade name Cannibal^{TM} and include an inert solid removal device (Chen et al., 2003; Goel and Noguera, 2006; Datta et al., 2009; Novak et al., 2003; Saby et al., 2003). A brief description of the process is presented here. A portion of the returned biomass is taken to an anaerobic sidestream reactor (fasting or anaerobiosis of sludge) and an equal volume of the mixed liquor from this sidestream reactor is sent back to the main bioreactor (feasting conditions). The circulation of biomass through the anaerobic sidestream to the main bioreactor causes a net reduction in the overall observed biomass yield.

Figure 1.3 depicts that one tenth of the underflow, which showed the maximum of solids destruction over the other methods (Easwaran, 2006), is going through the sidestream and the rest is bypassing this sidestream in the form of returned activated sludge. Likewise, one tenth of the mixed liquor from the sidestream tank is sent back to the main bioreactor. Cycling of a portion (one tenth in this case) of the secondary clarifier underflow through the anaerobic sidestream tank induces certain conditions (not known fully) under which the process depicted in Figure 1.3 achieves a net reduction (up to 60 %) in the biomass with synthetic wastewater (Datta et al., 2009; Goel and Noguera, 2006, Novak et al., 2007; Chon et al., 2011). However, the performance of sludge reduction has shown some inconsistencies in several full-scale sludge minimizing ASP plants coupled with Cannibal^{TM}. For example, the same configuration, primarily achieving

chemical oxygen demand (COD) removal, achieves reasonabe sludge reduction at one location and less sludge reduction at another location. It could be due to differences in influent characteristics.

Sheridon and Curtis (2004) proposed that the sidestream tank developed equilibrium between selection and destruction in the side stream bioreactor. That is, aerobic bacteria are selectively destroyed in the side stream reactor, then facultative bacteria break down and use the remains of the aerobes and their byproducts. When the mixed liquor is recycled back to the mainstream reactor, the facultative bacteria are out-competed by the aerobic bacteria and subsequently are broken down, which means a low yield has been obtained in the alternative environments of the aerobic treatment process and in the sidestream bioreactor. Park et al. (2006) proposed that a primary mechanism for the degradation of waste activated sludge under anaerobic conditions. The same mechanism was thought to apply to the Cannibal^{TM} system by Novak et al. (2006). That is, when thickened sludge is cycled to the anaerobic bioreactor, iron is reduced, and organic matter is released or solubilized. When the sludge and solubilized organic matter are returned to the aerobic bioreactor (activated sludge aeration basin), it is degraded rapidly, before it can be coagulated by iron in the sludge.

In summary, various mechanisms have been proposed minimize sludge in ASPs operating in fasting and feasting modes with no definitive answers. The information on the microbial community dynamics in ASPs aimed at sludge reduction is completely missing. This is important, because the information on microbial community can help designers and practitioners optimize the process. Furthermore, it also helps to understand why these configurations achieve sludge reduction.

Biological Nutrients Removal

The main nutrients of concern are nitrogen and phosphorus. Biological nitrogen removal requires nitrification and denitrification processes to be incorporated in the treatment train through the operation of oxic and anoxic zones. Nitrification is the oxidation of reduced forms of nitrogen, ultimately to nitrate. In aerobic conditions, the ammonia oxidizing bacteria (AOB) are able to oxidize ammonium to hydroxylamine by a membrane-bound ammonia monooxygenase, and then oxidize hydroxylamine to nitrite (Prosser, 2005) (as shown in equation (1)). *Nitrosomonas* and *Nitrosospira* are the major genera of AOB that are usually found in the activated sludge (Ma et al., 2015). Nitrite oxidizing bacteria (NOB) then further oxidize nitrite, from the last step, to nitrate (Prosser, 2005) (equation (2)). Small amounts of the NOB *Nitrospira* and *Nitrobacter* were detected in ASPs (Prosser, 2005; Siripong and Rittmann, 2007). After nitrification, denitrifiers such as *Pseudomonas*, *Alcaligenes*, *Paracoccus,* and *Thiobacillus* can convert nitrate to nitrogen gas via multiple steps with the additional carbon source (equation (3)).

$$NH_4^+ + O_2 + H^+ \rightarrow NH_2OH + H_2O \rightarrow NO_2^- \text{ (1)}$$

$$NO_2^- + O_2 \rightarrow NO_3^- \text{ (2)}$$

$$NO_3^- \rightarrow NO_2^- \rightarrow NO \rightarrow N_2O \rightarrow N_2 \text{ (3)}$$

Coupling sludge minimization with biological phosphorus removal through an enhanced biological phosphorus removal (EBPR) can be challenging and more complex. During the anaerobic condition, PAOs can convert short chain volatile fatty acids into polyhydroxyalkanoates (PHAs), with internal polyphosphate and glycogen reserves hydrolyzed to supply energy and reducing power to the cells, respectively (He et al., 2010). In the following aerobic step, PAOs use the stored PHAs and P is taken up from

the bulk liquid to form polyphosphate, simultaneously rebuilding their glycogen. Under the anaerobic and aerobic conditions, PAOs can take up more inorganic phosphate than their metabolic demand and store it as polyphosphate. The reduction of biomass from the treatment train at the end of the aerobic phase contributes to net P removal from the system.

Different configurations of anaerobic-anoxic-oxic (A^2O) were applied in lab-scale and full-scale bioreactors with efficient biological nutrient removal (Liu et al., 2013; van Loosdrecht et al., 1998). The anaerobic phase is used to release P and consume organics. Then the subsequent aerobic phase will enable P uptake and ammonia and organics oxidation. Finally, the last anoxic time period is provided to allow any denitrification. The information about the nutrient removal was absent in the sludge minimization process. It is feasible for an operation to significantly minimize sludge production via feasting and fasting without significantly impacting nitrogen removal, if the adequate key microorganisms remain viable. However, this becomes a challenge for EBPR, because the successful operation of EBPR requires the process to be operated at a finite SRT, typically at 5-15 days (Rodrigo et al. 1996; Shao et al., 1992; Fukase et al. 1985). Hence, under the conventional paradigm, successful EBPR requires biomass wastage. That means the ASP needs to be operated at a certain SRT to accomplish successful EBPR. As demonstrated in Figure 1.3 before, sludge minimizing ASP processes using biomass fasting and feasting approach essentially operate at nearly infinite or very high SRT which may not be suitable for EBPR.

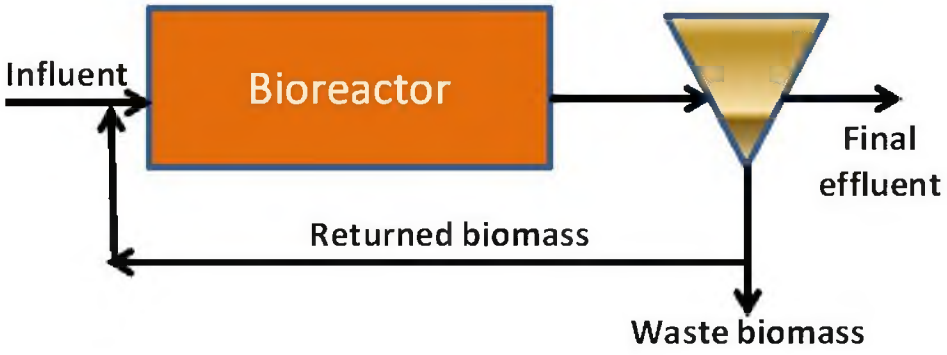

Figure 1.1: Typical configuration of activated sludge bioreactor

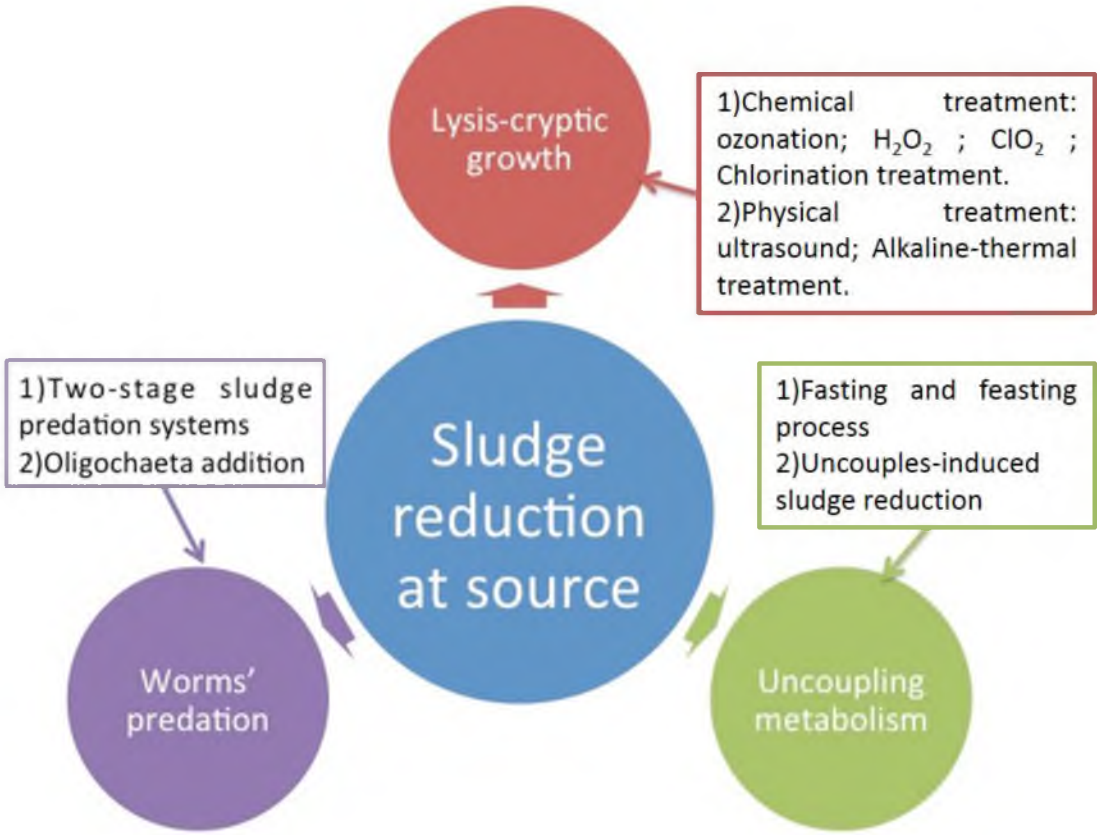

Figure 1.2: Outline of sludge reduction at source technologies

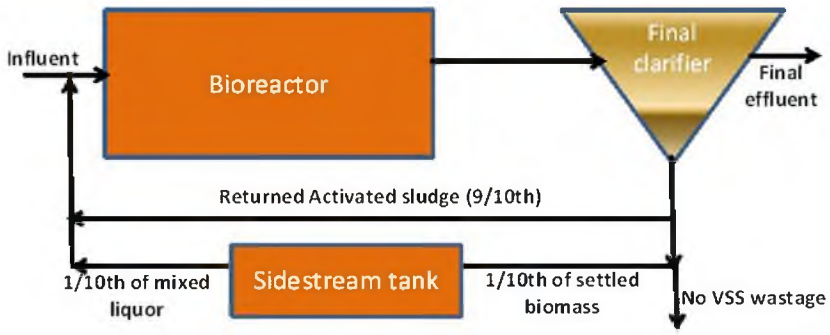

Figure 1.3: A schematic of a typical sludge minimizing activated sludge process through returned biomass fasting (in the sidestream tank) and feasting (in the bioreactor). Examples include the oxic settling anoxic process (primarily lab or pilot scale) and the Cannibal^{TM} process (some full scale applications).

RESEARCH HYPOTHESES AND OBJECTIVES

The overall objective of this study was to investigate the feasibility of coupling sludge minimization through fasting and feasting with nutrient removal. The following hypotheses have been formulated based on these challenges.

Hypothesis 1: Nutrient removal, especially biological phosphorus, can be sustained in sludge minimizing activated sludge processes, provided these that processes are operated at small biomass wastage rates, but still achieving appreciable biomass reduction.

Hypothesis 2: Sludge reduction is related to inert materials that are present in the influent and not hydrolyzed in the main bioreactor. The sidestream tank enhances improved hydrolysis of inert materials, compared to conventional configurations.

Hypothesis 3: The microbial community, including polyphosphate accumulating organisms and nitrifying bacteria in the sludge minimizing reactor, will be different from those in a conventional reactor run under the same conditions. Additionally, there were specific objectives that govern this research.

Objective 1: Operate two SBRs fed with synthetic influent to achieve simultaneous sludge minimization and nutrient removal. One SBR will be run in the sludge minimization mode (called the modified-SBR) and the second will be run as a control-SBR.

Task 1: Start-up the reactors using synthetic feed.

Task 2: Compare the sludge yield in both SBRs.

Task 3: Conduct carbon mass balance in these two SBRs using ^{13}C carbon substrate.

Task 4: Conduct cost/energy estimation between this sludge reduction process and the conventional sludge handling processes.

Objective 2: Evaluate the effect of the presence of inert organics in the influent on the sludge yield by changing the feed from synthetic to real wastewater.

Task 5: Change feed to real wastewater in stepwise manner.

Task 6: Document the response in terms of performance and sludge yield.

Objective 3: To study the ecology of key microorganisms using conventional molecular analysis tools and the overall ecology using the high throughput metagenomics in lab scale reactors operating under Objectives 1 and 2.

Task 7: Study the ecology of ammonia oxidizing bacteria (AOB), nitrite oxidizing bacteria (NOB), polyphosphate accumulating organisms (PAOs) and denitrifiers.

Task 8: Study the overall microbial ecology using metagenomics analysis (Illumina Miseq).

CARBON MASS BALANCE AND MICROBIAL ECOLOGY IN A LABORATORY SCALE REACTOR ACHIEVING SIMULTANEOUS SLUDGE REDUCTION AND NUTRIENT REMOVAL*

Abstract

Biomass reduction in activated sludge processes (ASP) at source using process manipulation has been researched widely over the last two decades. However, the absence of nutrient removal component, lack of understanding on the organic carbon, and limited information on key microbial community in biomass minimizing ASP preclude the widespread acceptance of sludge minimizing processes. In this manuscript, we report simultaneous biomass reduction through anaerobiosis along with nitrogen and phosphorus removals. The manuscript also reports carbon mass balance using stable isotope of carbon, microbial ecology of nitrifiers and polyphosphate accumulating organisms (PAOs). Two laboratory scale reactors were run in anaerobic-aerobic-anoxic (A^2O) mode. One reactor was run in the standard mode (hereafter called the control-SBR) simulating conventional A^2O type of activated sludge process and the second reactor was run in the sludge minimizing mode (called the modified-SBR). Unlike in other research efforts where sludge minimizing reactor was run at nearly infinite solid retention time

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(SRT) and, to sustain the efficient nutrient removal, the modified-SBR in this research was operated at a very small biomass yield rather than at infinite SRT. Both reactors showed consistent NH_4^+-N, phosphorus and COD removals over a period of 263 days. Both reactors also showed active denitrification during the anoxic phase even if there was no organic carbon source available during this phase suggesting the possible presence of denitrifying PAOs (DNPAOs). The observed biomass yield in the modified-SBR was 60 % less than the observed biomass yield in the control-SBR. The modified-SBR showed the greater diversity of ammonia oxidizing bacteria and PAOs than in the control-SBR. The diversity of PAOs in the modified-SBR was even more interesting in which case novel clades of *Candidatus* Accumulibacter phosphatis, an uncultured but widely found PAOs, were found in the modified-SBR.

Introduction

Activated sludge process is the most widely used treatment method for municipal wastewater (Grady et al., 1999; Metcalf and Eddy, 2003). However, excess sludge is the one of the main drawbacks of the activated sludge process. Treatment of this excess sludge requires much energy and labor. Sludge reduction through process manipulation at wastewater treatment plants is increasingly attractive due to rising costs and constraints associated with sludge treatment and disposal. The treatment of excess sludge is expensive and may account for 25 to 65% of the plant's operational cost (Chen et al., 2001; Camacho et al., 2002; Saby et. al., 2003; Cui and Jahng, 2004). Approximately 8.2 million tons of sludge was generated in 2010 in the United States, and the amount has been predicted to continue to grow (USEPA, 1999).

Anaerobic digestion reduces the excess biomass by 40~50 % with methane gas

being a useful byproduct, albeit it is a green house gas. Several research efforts have also shown that electricity (Liu et al., 2004, Min and Logan., 2004) and hydrogen gas (Angenent et al., 2004; Hallenbeck., 2005 and Gong et al., 2005) can be generated biologically from the wasted biomass. However, challenges still exist regarding the improved yields of electricity and hydrogen gas using microbial fuel cell and biomass fermentation biotechniques, respectively. Other option for the use of biomass includes its composting followed by land application. However, land application of biosolids is restricted in many states due to the health risk to man and livestock owing to potentially toxic elements in the sewage sludge, i.e., heavy metals, pathogens, persistent organic pollutants, and nutrients (Wei et al., 2003). Hence, it is highly debatable that excess biomass is a useful commodity. As a result, excess biomass from activated sludge processes is regarded as an environmental concern and threatens the sustainability of activated sludge treatment processes. The reduction in sludge could dramatically impact the difficulties municipalities are facing today in disposing of or reusing their excess sludge.

For sludge reduction at the source, a number of technologies have been developed that are one or a combination of physical, chemical, biological, and thermal processes (reviewed by Ødegaard, 2004). However, cost savings from sludge minimization using one or a combination of physical, chemical, and thermal processes (Figure 3.1A) must be compared to costs involved in implementing these processes. All these alternatives are expensive and could increase the overall energy consumption of the plant (Böhler and Siegrist, 2006).

Sludge minimization through anaerobiosis (also called the fasting of biomass, as

shown in Figure 3.1B) of returned activated sludge using a sidestream anaerobic reactor is relatively new sludge minimization approach which has been primarily investigated in laboratory scale setups with few full scale installations in the U.S under the trade name of Cannibal™. In this approach, a portion of the settled biomass is taken to an anaerobic sidestream reactor (fasting of sludge) and an equal volume of the mixed liquor from this sidestream reactor is sent back to the main bioreactor (feasting conditions). The circulation of biomass through the anaerobic sidestream to the main bioreactor causes a net reduction in the overall observed biomass yield. Cycling of a portion of the secondary clarifier underflow through the anaerobic sidestream tank induces certain conditions (not fully known) under which the process achieves a net reduction in the biomass.

Despite many significant advantages, several factors preclude the widespread application of activated sludge configurations which achieve a net sludge reduction through anaerobiosis. These factors include: (1) the lack of proven mechanisms of sludge reduction in these processes although some theories like iron is reduced and proteins are released and solubilized in the sidestream reactor were proposed (Novak et al., 2006), (2) the absence of the information on the fate of carbon, i.e., lack of carbon mass balance and, (3) (most importantly), the absence of nutrient removal component in these processes. From wastewater operator's perspective, the first two factors may not be too important as long as the process works efficiently. The last one is more essential because nutrient removal is mandated by federal and state regulatory agencies to protect the quality of receiving waters and, consulting world does not have a sound design basis of these sludge minimizing processes.

Main nutrients of concern are nitrogen and phosphorus. Biological nitrogen

removal requires nitrification and denitrification processes to be incorporated in the treatment train through the operation of oxic and anoxic zones not necessarily in this order. It is feasible for an operation to significantly minimize sludge production via feasting and fasting without significantly impacting nitrogen removal if the adequate key microorganisms remain viable. Coupling sludge minimization with biological phosphorus removal through enhanced biological phosphorus removal (EBPR) is challenging and more complex. Activated sludge processes achieving sludge minimization using anaerobiosis (fasting and feasting) have been operated at nearly infinite or very high solids retention time (SRT) (Goel and Neguera, 2006; Novak et al., 2006; Datta et al., 2009). This becomes a challenge, especially for EBPR, because the successful operation of EBPR requires the process to be operated at a finite SRT, typically at 5-15 days (Fukase et al., 1985; Shao et al., 1992; Rodrigo et al., 1996).

In this chapter, we report some key and important findings related to the sludge minimizing ASP. We employed a stable isotope approach to accomplish carbon mass balance in the sludge minimizing lab scale reactor. The ecology of nitrite oxidizing bacteria, denitrifiers based on 16S rDNA, and the diversity of AOBs were used *amo*A genes. This chapter reports these important research findings.

Materials and Methods

Reactor Operation

Two sequencing batch reactors as shown in Figure 3.2 were operated: one in the conventional mode (called the control-SBR-left one in the Figure 3.2) and the other in the sludge minimization mode (called the modified-SBR-right one in the Figure 3.2) using returned biomass fasting and feasting strategy for 263 days to study carbon mass balance

and to investigate the ecology of PAOs, ammonia and nitrite oxidizers. The control-SBR was run at 10-days SRT and the modified-SBR was run in sludge minimization mode (Figure 3.2). The modified-SBR was run at infinite SRT at the beginning and as a result, the solids accumulated in this SBR. The rate of solids accumulation (i.e., observed biomass yield) was calculated in the modified-SBR and thereafter, the modified-SBR was operated at the observed yield at which the solids accumulated in the modified-SBR. In this case, the biomass equivalent to the calculated observed yield was directly wasted from the SBR.

The cycle of each SBR consisted of 5.5 h of reaction period in A^2O mode, the distribution of which was as follows; (1) 1.5 h fill and anaerobic, (2) 2.5 h aerobic, (3) 1.5 h anoxic followed by, (4) 30-min settling and decant. Other operational details and feed composition was similar to the one used by Datta et al., (2009).

To induce fasting and feasting of the returned biomass in the modified-SBR, one tenth of the settled biomass from the modified-SBR at the end of each cycle was brought to a sidestream reactor and this sidestream reactor was designated as the modified holding tank (MHT). Following this, one tenth of the mixed liquor from this sidestream was recycled back to the modified-SBR at the beginning of each cycle. Recycling of one tenth of biomass back and forth enabled an overall internal SRT of 10-days. Furthermore, as stated before, the modified reactor was run at "small biomass wastage" rather than at an infinite SRT to sustain efficient EBPR and to avoid any biomass buildup in the reactor system. On the other hand, one tenth of the settled biomass from the control-SBR was taken to a conventional anaerobic digester operated at a 10-day HRT and the observed biomass yield in the control-SBR was calculated after the biomass was digested in the

conventional digester. The conventional digester associated with the control-SBR was termed as the control holding tank (CHT). Both SBRs were monitored for inorganic nutrient species. For yield calculation purpose, the cumulative wastage in terms of sampling wastage and solids present in the final effluent were also considered.

Reactor performance was monitored in terms of dissolved COD, phosphorus (P) and ammonia removals and the biomass yield was calculated based on total suspended solids (TSS)/volatile suspended solids (VSS) concentrations measured in SBRs, digester and sidestream and effluents. Observed yield was determined by Metcalf and Eddy (2004), which was the ratio of the amount of biomass produced to the amount of substrate consumed. In this study, the observed yield was determined over a given range of operation as the VSS increase/COD used, using all the data over the range of operation for which the yield was calculated. The cumulative wastage was calculated from sampling wastage, effluent wastage, and average biomass growth.

CO_2 Formation Rates Using ^{12}C and ^{13}C Substrate

To evaluate the CO_2 formation rates, a known volume of mixed liquor from each SBR was taken in 70-mL serum bottles. The mixed liquor was spiked with the ^{12}C carbon substrate and the serum bottle was sealed airtight. The air present in the head space was assumed to be sufficient to support the aerobic growth of bacteria. Samples bottles were analyzed at zero and 4 h to calculate the initial and final moles of CO_2 present in the head space. The CO_2 generated as a result of various biogeochemical activities in the biomass was calculated from the difference of these two values. The final concentration of CO_2 was expressed in terms of moles of CO_2 $g^{-1}VSS^{-1}$ to account for differences in VSS contents in both SBRs. During each analysis, the mixed liquor was acidified with HCl to

make sure that all the dissolved CO_2 was accounted for during the analysis. In the headspace, triple 0.2 mL standard gas was injected for the purpose of calibration in the beginning, the unknown CO_2 percentage was determined by comparing with the known standard gas using ideal gas law (PV=nRT).

Carbon mass balance was performed on both SBRs, the procedure was the same as the CO_2 formation experiment mentioned above, except that the two of the bottles were spiked with acetate with 20% of two of the C atoms being ^{13}C and other micronutrients. These two spiked serum bottles were termed as sample bottles. The other two bottles with no spiking were termed as control bottles. One bottle from each of sample and control bottle sets was immediately analyzed for ^{13}C in the head space gas and the biomass.

For head space gas analysis, a standard GC with Delta Plus isotope ratio mass spectrometer (Finnigan-MAT, Bremen Germany) was employed. A Poraplot QC column with 3-m effective length was used. For direct gas analysis, 0.2 mL gas sample was injected into a modified Elemental Analyzer (model 1110, Carla Erba, Milan, Italy) which was connected to the GC through an open spit interface and using a FinniganConflo III interface. For biomass samples, 1 mg homogenized dry biomass (dried overnight at 103^{o}C) was combusted in the presence of oxygen. Water vapors which may have been present during combustion were removed using chemical trap. Stable isotope ratios for laboratory reference materials were calibrated using NBS-19 for carbon. The standard deviations (SD) of repeated measurements of the same commercially produced powdered keratin reference material throughout all analyses were 0.2 for carbon.

The atom percentage (AT%^{13}C) was used to calculate ^{13}C concentration in the

total moles of CO_2. The percentage recovery of ^{13}C was determined by dividing the total mass of ^{13}C partitioned into the gas phase and the biomass divided by the total spiked mass of ^{13}C in the form of acetate. In all calculations, background ^{13}C present was accounted for by analyzing blank samples.

Microbial Ecology in the Reactors

DNA Extraction, PCR, and terminal restricted fragment length polymorphism (TRFLP) for ammonia and nitrite oxidizers. Genomic DNA was extracted from biomass samples collected from the reactors using a soil DNA extraction kit (MoBio Labs, Solana Beach, CA). TRFLP for AOBs was performed using the modified protocol developed by Park and Noguera (2004). Restricted enzyme digested fragments were processed on an Applied Biosystems 3730 Genetic Analyzer capillary electrophoresis instrument (Applied Biosystems, Foster City, CA) at the University of Utah Core Facility and analyzed using the GeneMapper software (Applied Biosystems, Foster City, CA) version 2.6. The resulting fragment lengths were compared with known fragment lengths of AOB to identify presence of specific AOB (Park and Noguera 2004, Park et al. 2002, Horz et al. 2000). In case of NOBs, TRFLP was performed using the modified protocol developed by Siripong and Rittmann (2007). For cloning, the 491bp *amoA* gene fragment for AOBs and 16S rRNA gene for NOBs were amplified using the same strategy and primers that was used for TRFLP except that the primers were not labeled.

Quantification of ammonia oxidizers. The qPCR was conducted on a Real plex Mastercycler (Eppendorf, NY) using iQ SYBR green supermix (Bio-Rad, Hercules, CA) with a total reaction volume of 20μL. Using amoA-1F and amoA-2R to round only one time PCR, to amplify the targeted 491-bp fragment according to the protocol used by

Park and Noguera, (2004), Racz et al. (2010). The thermal profile worked for amplification of amoA gene sequence is as follows: denaturation at 94°C for 5 min, followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 56°C for 1.5 min, and elongation at 72°C for 1.5 min, with polishing steps at 60°C for 1.5 min and 72°C for 10 min.

PCR for PAOs using 16S rDNA and *ppk1* gene targeted primers PAO ecology was targeted by employing cloning and sequencing using Candidatus Accumulibacter phosphatis, an uncultured PAO, specific (Crocetti et al., 2000) and ppk1 gene specific biomarkers. In both cases, genomic DNA was extracted and purified as described earlier. To amplify 16S rDNA region specific to Candidatus Accumulibacter phosphatis-related PAOs in *Rhodocyclus* family, the forward primer, PAO651f (5'-CTGGAGTTTGGCAGAGGG-3') (Hesselmann et al., 1999) and a reverse universal eubacterial primer (1492r 5'-GGYTACCTTGTTACGACTT-3') (Lane DJ., 1991) were used. Amplification was performed using the temperature program described in Zilles et al. (2002), except that the annealing temperature was adjusted to 58°C. To amplify *ppk1* gene, ACC*ppk1*-254F: 5`-TCACCACCGACGGCAAGAC-3`as the forward primer and ACC*ppk1*-1376R: 5`-ACGATCATCAGCATCTTGGC-3' as the reverse primer were used (McMahon et al., 2007). The reaction mixture contained 1X GoTaq PCR buffer (Promega, WI), 3.0 mM $MgCl_2$, 200 µM of each dNTP, 400 nM each of forward and reverse primer, 5% of DMSO, and 0.05 U/µl of GoTaq DNA polymerase (Promega, WI). The PCR was conducted on a Gradient Mastercycler (Eppendorf, NY), with the program consisting of 10-min initial denaturation step at 95°C, followed by 30 cycles at 95°C for 30s, 68°C for 60s and 72°C for 120s, and followed by a final extension at 72°C for 12-

min.

In all cases, PCR products were first verified on 1% agarose gel, subsequently gel purified using a Qiaex II gel extraction kit (Qiagen, Valencia, CA). The gel purified amplicons from the control-SBR and modified-SBRs were cloned, screened, and sequenced as described below.

Construction of clone libraries. In each case, the purified PCR amplicons were cloned into pCR4 of TOPO TA Cloning Kit (Invitrogen, CA) following manufacturer's instructions. Clones from each library were screened for the presence of insert using the clone-PCR with corresponding primers. Plasmid DNA from these clones were extracted using Zippy kit (Zymo Research, CA) and sequenced at DNA sequencing Core facilityat the University of Utah. Chimera check (Bellerophon) was performed on the sequences prior to the phylogenetic analysis (Huber et al., 2004). In case of 16S rDNA, the retrieved sequences were compared for homology using RDP II (Cole et al., 2007) and NCBI BLAST (Altschul et al., 1990). Phylogenetic tree was constructed using MEGA version 6 software (Tamura et al., 2007). Preliminary operational taxonomic units (OTUs) were defined based on 98% sequence homology between retrieved sequences.

Analytical Methods

Samples were routinely collected at the end of each period, filtered (0.45μm) and analyzed. Chemical oxygen demand (COD), ammonia (NH_4^+-N), nitrate (NO_3-N), nitrite (NO_2-N), and dissolved phosphorus (PO_4^{3-}-P), were quantified using HACH methods 8000, 10031 (Salicylate method), 10020 (Chromotropic Acid method), and 8153 (Ferrous Sulfate method), 8048 (Ascorbic Acid method), respectively. The mixed liquor solids concentration was determined as total suspended solids (TSS) and as volatile suspended

solids (VSS), both were measured in accordance with Standard Methods (APHA, 1985). Sludge volume index (SVI) was determined using the method 2710D in Standard Methods (APHA, 1985).

Results

Reactor Performance in Terms of Nutrient Removal

Figure 3.3 shows performances of both SBRs in terms of phosphorus removal. The average dissolved PO_4^{3-}-P released at the end of the anaerobic phase was 13.82±1.95 mg PO_4^{3-}-PL^{-1}in the control-SBR and 17.65±3.10 mg PO_4^{3-}-P L^{-1}in the modified-SBR, respectively. The dissolved PO_4^{3-}-P in the final effluent from both SBRs was always below 1 mg PO_4^{3-}-P L^{-1}. Overall, both SBRs consistently showed more than 85 % PO_4^{3-}-P removal efficiency. Most of the COD was consumed by the end of the anaerobic phase during each cycle in both SBRs and nearly complete COD removal (graphs not included) was consistently recorded in both SBRs.

Figure 3.4 shows reactor performance for NH_4^+-N removal. The average NH_4^+-N in the influent to both SBRs during the operational period was 26.73±1.4 mg NH_4^+-NL^{-1}. It is evident from this figure that the NH_4^+-N concentration in the final effluents of both SBRs was below detection limits. The difference in the NH_4^+-N concentration in the influent and at the end of anaerobic phase was primarily due to the dilution effect in which case, the incoming influent (667 mL) at the beginning of each cycle was mixed with the mixed liquor (1333 mL) which was already present in the reactor at the beginning of each cycle.

Figure 3.5 (a and b) shows NO_2-N concentrations in both SBRs at specified time periods. In the control-SBR, periodic episodes where NO_2-N as high as 4 mgL^{-1} NO_2-N

was recorded at some occasions, and were observed, for example on 50-, 80- and 150th day (Figure 3.5a). In between, the average NO_2-N concentration at the end of aerobic cycle in the control-SBR was 1.63±0.89 mgL^{-1}. On the other hand, except on a few occasions, NO_2-N concentrations at the end of the aerobic cycles in the modified-SBR were always below detection limits. In general, the rise in NO_2-N concentrations in the mixed liquor in both SBRs corresponded to drops in NO_3-N concentrations (Figure 3.6a and b). The average NO_3- N concentrations at the end of the aerobic cycle in the control-SBR and the modified-SBR were 5.04± 0.89 and 5.82 ± 0.84 mgL^{-1}, respectively.

Reactor Performance in Terms Biomass Yield

Figure 3.7 shows TSS and VSS in the control-SBR (panel a) and the modified-SBR (panel b). The average TSS and VSS concentrations in the control-SBR were 3342±497 and 2984±436 mgL^{-1}, respectively. Likewise, the average TSS and VSS concentrations in the modified-SBR from the beginning to the 120th day were 4068±726 and 3096±285 mgL^{-1}, and 2901±463 and 2128±195 mgL^{-1} from the 135th to the 263th day respectively. Solids built up in the modified-SBR from the 120th day until the 135th day because of the malfunctioning in the recycling pump which was pumping one tenth of the settled biomass from the modified-SBR to the sidestream reactor. On the 135th day, to avoid solids going out with the effluent, a known volume of the settled biomass from the modified-SBR was transferred to the sidestream reactor attached to the modified-SBR, and thereafter, the solids profile in this SBR dropped and followed a steady state trend.

Overall, sludge in the modified-SBR had a volatile fraction of approximately 0.74, which agrees with the previous result around 0.65 to 0.7 (Easwaran and Novak, 2006). This lower volatile fraction indicates the loss of VSS in similar system and might indicate

that iron accumulated in the sludge (Novak at al., 2007). The ratio is likely due to the stabilization of the organic fraction in the reactor. Otherwise, the average of VSS to TSS ratio in the control-SBR was 0.90, which was higher than the modified-SBR.

Figure 3.8 shows linear regression that was performed on cumulative solids and COD to calculate biomass yields. The cumulative solids expressed in terms of total mass of gVSS accounted for VSS losses due to sampling and in the effluents of both SBRs. The cumulative VSS line for the modified-SBR system, which consisted of the SBR part and the sidestream reactor, is represented by gray circles. Based on the linear fit, the observed biomass yield was estimated to be 0.114 mgVSS mg^{-1} COD^{-1}. Similarly, the observed biomass yield for the control-SBR system for which the regression line fit is represented by black circles in Figure 3.8 was estimated to be 0.333 mg VSS mg^{-1} COD^{-1}. Hence, the overall observed biomass yield in the modified-SBR was almost 60 % less than the yield in the control-SBR system. It is worth mentioning that the calculated observed biomass yield for the control-SBR included the digestion of biomass in the attached conventional digester referred as control holding tank (CHT).

CO_2 Formation Rates and Carbon Mass Balance in Both SBRs

Based on the ideal gas laws and biomass concentrations in serum bottles, the specific molar concentrations of CO_2 in serum bottle head space were calculated for an experimental period of 4 h. Based on the calculations, the mixed liquor from the modified-SBR enabled almost 44 % more CO_2 based on specific CO_2 formation rates than the mixed liquor from the control SBR. These tests were repeated and the results were consistent. Based on these experiments, it can be concluded that the modified-SBR mixed liquor poses greater capacity than the mixed liquor in the control-SBR to

mineralize the organics to CO_2 at a faster rate.

CO_2 formation experiments under controlled conditions do not provide information on the partition of carbon substrate into the gas phage and the biomass. Hence, serum bottle tests were repeated but ^{13}C labeled acetate was used this time. In these ^{13}C spiked experiments, an attempt was made to achieve carbon mass balance. Based on the total mass of the spiked ^{13}C, ^{13}C in the head space and in the biomass, it was estimated that almost 41.5 % of the total spiked ^{13}C mass partitioned to form new biomass in the case of the mixed liquor from the control-SBR where as it was about 29.5 % for the modified-SBR mixed liquor. Head space analysis of ^{13}C confirmed that nearly 56.5 % and 74 % of the total spiked ^{13}C went into the head space in the form of CO_2 gas for the control-SBR and the modified-SBR, respectively. These results further support the notion that less biomass in the modified-SBR was generated as a result of efficient degradation of organics to gaseous byproducts. Furthermore, results also answered the question why the modified-SBR had low biomass yield although these results did not shed light on the mechanisms of low biomass in the sludge minimizing modified-SBR.

Microbial Community Composition

Ammonia and nitrite oxidizing community using TRFLP. Ammonia oxidizing bacterial community was studied using *amoA* gene targeted TRFLP. The *amoA* gene codes for ammonia monooxygenase which catalyzes oxidation of ammonia to nitrite and previous studies have developed TRFLP targeting *amoA* gene (Park and Noguera, 2004; Siripong and Rittmann, 2007). Based on the terminal fragment analysis (Figure 3.9), the control-SBR was found to be dominated by AOBs belonging to *Nitrosomonas oligotropha* lineage (terminal fragments: 48/135). In the modified-SBR, much diverse

AOB community belonging to *Nitrosomonas oligotropha* (terminal fragments: 48/135. 354/135), *Nitrosomonas cyrotolerans and Nitrosomonas marina* (terminal fragments: 48/441) lineages were recorded. Quantification of *amoA* gene in both SBRs using qPCR inferred that 1.52E-03±8.71E-05 copies/ng DNA and 1.39E-3±1.63E-04 copies/ng DNA were present in control-SBR and modified-SBR, respectively. Greater diversity of AOBs was in the modified-SBR but presence of comparable copy numbers of *amoA* genes in both the reactors indicate the existence of equivalent AOB populations resulting in similar NH_4^+-N removal observed in both the reactors (Figure 3.4).

Two widely known and well studied nitrite oxidizers belong to *Nitrospira* and *Nitrobacter* genus. Terminal fragment analysis using *Nitrospira* and *Nitrobacter* specific primers indicated that both control-SBR and modified-SBR harbored the NOB genera belonging to *Nitrospira* (TF sizes 277 and 333) (Figure 3.10) and *Nitrobacter* (TF 141) (Figure 3.11). In addition, control-SBR presented weak signal around TF sizes of 134 and 194. These findings agree with previous studies where *Nitrospira*-like bacteria were the dominant NOB in both full-scale wastewater treatment plans and lab-scale reactors (Maixner et al. 2006).

Phylogenetic classification of AOBs and NOBs. The TRFLP technique just provided qualitative information on the presence or absence of the AOBs and NOBs. However, TRFLP did not reveal the genetic diversity in the respective general that was indicated to be present based on the TRFLP profiles. In order to get a finer scale resolution in AOBs and NOBs community present in both reactors, aomA gene and 16S based cloning and sequencing was performed on the genomic DNA which was obtained from both reactors.

The phylogenetic analysis based on amoA gene sequences from both reactors is depicted in Figure 3.12. All of the clones from control-reactor were found to be associated with uncultured *Nitrosomonadaceae* bacterium within the *N. oligotropha* lineage. The uncultured *Nitrosomonadaceae* bacterium to which all the clones from control-SBR matched with was found within rhizosphere samples recovered from a lab-scale constructed wetland (Kikolausz et al., 2004). Half of the clones (12 out of 22) from the modified-SBR had sequence similarity (98%) with another uncultured bacterium recovered from granular activated carbon (GAC), which was used for advanced drinking water purification (Kasuga et al., 2011). The rest of the 10 clones from modified-SBR had greater similarity (99%) with uncultured bacterium which was found in the Pearl River estuary water column in China (Zhu and Fan, 2010).

Clones recovered using primers targeting NOBs related to *Nitrospira* genus in both reactors is shown in Figure 3.13. Nitrospira was found with over 95% homologus to Candidatus *Nitrospira defluvii* (Genoscope, 2010) and one *Nitrospira* sp was in the activated sludge from Japan (Fujitani et al., 2014). On the other hand using biomarker NIT3, no clones were found to be homologus to *Nitrobacter* (Figure 3.14). One clone in control-SBR (OTU1) was closely associated with the *Afipia clevalandensis* from activated sludge (Hashimoto et al., 2009). OUT2 (6 clones from modified-SBR) and OTU3 (5 clones from control-SBR, 11 clones from modified-SBR) was mainly associated with uncultured *Bradyrhizobiaceae* bacterium. This uncultured bacterium was found in soils, and can fix nitrogen (Freitag et al., 2005). Two clones in modified-SBR formed OTU4 with the 98% similarity with *Rhodopseudomonas* sp., which was found in the sediment from Fenhe River, China (Zhan et al., 2014). Approximately 70% and 20%

of the clones in control-SBR and modified-SBR had over 90% similarity with uncultured bacterium and closest genus was found to be *Mesorhizobium sp.* which is known as denitrifying bacterium (Yoshie et al., 2004).

PAO community using Candidatus Accumulibacter specific 16S rDNA biomarkers. Figure 3.15 shows a phylogram obtained after aligning the partial 16S rDNA sequences using *Candidatus* Accumulibacter phosphatis specific primers and other related sequences obtained from publicly available databases. From the Figure 3.15, it can be concluded that there are three distinct divisions across which all clones belonging to the control and the modified SBR are distributed. The top portion of the phylogram covers all clades I, IIA, IIB, IIC, and IID belonging to *Candidatus* Accumulibacter phosphatis (He et al., 2007; Kim et al., 2010) and almost 70% of the clones from both reactors were distributed in this region of the phylogram. Interestingly, 18% of the clones from the modified-SBR formed separate cluster in the middle of the phylogram and emphasizes the fact that diversity within *Candidatus* Accumulibacter related populations and PAOs in general is much greater in the modified-SBR than in the control-SBR. Besides this, there was a lineage with clones from the control-SBR, which were more closely related to *Rhodocyclus tenuis* and formed a cluster towards the bottom of the phylogram. Although all sequences were obtained using *Candidatus* Accumulibacter specific primer PAO651 (Crocetti et al., 2000), some of the clones, especially from the control-SBR were not related to *Candidatus* Accumulibacter. This raises the question about the specificity of PAO651 towards all "*Candidatus* Accumulibacter" related PAOs (McMahon et al., 2002).

Microbial diversity based on Accumulibacter-like ppk1 genes: Polyphosphate

kinase subunit 1 is encoded by ppk1 gene and has been used previously to target PAO diversity (He et al., 2007). The 16S rDNA based clone library established the fact that there is a greater diversity of PAOs within *Candidatus* Accumulibacter phosphatis lineage. To further explore the fact and to confirm the diversity, the *ppk1* gene was constructed and analyzed based on the clone-library. Figure 3.16 shows the phylogram obtained by comparing the retrieved *ppk1* fragments with those taken from publicly available databases. The phylogram shows the distribution of *ppk1*clones retrieved from the two reactors along with other *ppk1* genes reported in previous studies (He et al., 2007; Kim et al., 2010). Although both reactors seem to harbor some common PAOs as was revealed using 16S rDNA clone library, there was a noticeable differences in the *ppk1* genes retrieved from both SBRs. Four distinct lineages emerged, suggesting the presence of much more diversity of PAOs than the diversity reported earlier. All *ppk1* genes obtained from control-SBR were confined in the clade IIA (He et al., 2007). However, *ppk1* genes from modified–SBR were affiliated to clades I, IIA, and two novel clades identified in this study. Interestingly, around 50% of these *ppk1*genes from the modified-SBR were found to fall under the novel clades.

Discussion

Reactor Performance

The reactors were run with synthetic feed. Since, the solids in the modified-SBR was not wasted initially, solids accumulated in this SBR. The solids accumulation rate (i.e., the overall observed biomass yield) in the modified-SBR was calculated and the biomass wastage was initiated at this rate from the modified-SBR. This marked the steady state of the modified-SBR and the beginning of this steady state is represented as

time zero in reactor performance graphs.

The P removal efficiencies in both reactors were always more than 85 %. The control-SBR was operated at a SRT of 10-days. Based on the small biomass wastage directly from the modified-SBR, the overall observed SRT for this SBR was calculated to be 175-days which is very high and beyond the optimum SRT value of 10 to 15-days recommended for efficient EBPR in the literature (Fukase et al., 1985; Shao et al., 1992; Rodrigo et al., 1996). Despite this high SRT, the sludge minimizing modified-SBR consistently showed efficient P removal. In a previous effort where the sludge minimizing bioreactor was run at an infinite SRT, the reactor initially showed efficient P removal but eventually failed after a period of 66 days (Goel and Noguera, 2006). In the present study, the sludge minimizing reactor was operated for more than 9 months using the new strategy of small biomass wastage and EBPR was stable in this reactor during the entire operational period.

Both SBRs showed very efficient NH_4^+-N removals. However, occasional NO_2-N buildup was recorded, especially in the control-SBR. The nitrification is a two-step process. In the first step, NH_4^+-N is first oxidized to NO_2-N. The step is catalyzed by ammonia oxidizing bacteria (AOBs). In the subsequent step, NO_2-N is oxidized to NO_3-N by nitrite oxidizing bacteria (NOB). In the overall NH_4^+-N oxidation, the first step, which is NH_4^+-N oxidation to NO_2-N, is the rate limiting (Wankel et al., 2011). Several factors including limited dissolved oxygen, pH and short hydraulic retention time can result in incomplete nitrification resulting in nitrite buildup. However, both SBR's were run under similar pH, HRT, and DO conditions. Although, the exact reason for difference in NO_2-N concentrations in both SBRs is not known, it may be possible that the nitrifying

population in the modified-SBR was more robust and diversified than the nitrifying population in the control-SBR. This hypothesis seems to be true in light of TRFLP results for AOBs for both SBRs in which case, the modified-SBR showed a greater diversity of AOBs than in the control-SBR.

The NO_2-N present at the end of aerobic phase was mostly denitrified during the anoxic phase in case of the modified-SBR, whereas occasional episodes of NO_2-N were observed in the control-SBR indicating inefficient denitrification during the anoxic phase in this SBR. The difference in NO_3-N concentration at the end of the aerobic cycle and at the end of the anoxic cycle indicates nitrate reduction possibly through biological denitrification (Figure 3.6a and b). As stated previously, COD was completely consumed during the anaerobic phase and aerobic phases leaving almost no appreciable carbon source for denitrification during the last anoxic phage. Hence, the possibility of biological nitrate reduction to reduced forms of nitrogen did not seem feasible. Denitrifying Polyphosphate-accumulating organisms (DNPAOs) (Saito et al., 2004) are a special class of PAOs which have recently caught attention of many researchers. DNPAOs use intracellular Polyhydroxyalkanoates (PHA) as a carbon source for denitrification. Although we did not investigate this aspect in detail, the possibility of the existence of DNPAOs in both SBRs cannot be ruled out. Furthermore, the modified-SBR was more efficient in NO_3-N reduction than the control-SBR. In fact, consistent NO_3-N removal in the modified-SBR was recorded after the 55^{th} day (except on the 151^{st} day).

Biomass Yield and Carbon Mass Balance

The modified-SBR was operated at an observed yield of 0.114 mg VSS mg^{-1} COD^{-1}. Unlike in many past studies at lab scale, the sludge minimizing modified-SBR was operated at this small biomass yield and the operation of this SBR was sustainable for nutrient removal and biomass reduction. This research demonstrated for the first time that operating a reactor in fasting and feasting mode (anaerobiosis) at small biomass yield rather than at no biomass wastage (infinite SRT) to achieve biomass reduction is not only sustainable but can also be combined with efficient nutrient removal.

The overall observed biomass yield in the modified-SBR system was almost 60 % less than the yield in the control-SBR. In the biomass yield calculations for the control-SBR, the biomass destruction through the conventional anaerobic digestion was also accounted for. Figure 3.8 also shows regression fit for the total biomass wasted from the control-SBR before it goes to the anaerobic digestion in CHT. This yield was estimated to be 0.651mg VSS mg^{-1} COD^{-1}. If we compare 0.333 mg VSS mg^{-1} COD^{-1} with 0.651 mg VSS mg^{-1} COD^{-1}, it can be concluded that the conventional digester CHT attached to the control-SBR helped to achieve almost a 49 % reduction in VSS and this reduction is in close proximity to the values reported for conventional anaerobic digestion (Novak et al., 2011). If we assume the same biomass yield, i.e., 0.651mg VSS mg^{-1} COD^{-1}, for the modified-SBR without the biomass going to the sidestream reactor referred as MHT and compare with the biomass yield (i.e., 0.114 mg VSS mg^{-1} COD^{-1}) when the MHT is attached to the modified-SBR, the overall reduction in the observed yield is almost 83%. This indicates that the mechanisms contributing to the biomass reduction in the modified-SBR through the attachment of the sidestream MHT are different from mechanisms

which exist in the conventional digester attached to the control-SBR.

There have been several efforts in the past aimed at sludge reduction primarily running the laboratory scale reactor at infinite SRT. However, none of the past efforts tried to show carbon mass balance using stable isotope of carbon. This is a fundamental question as why do sludge-minimizing reactors such as the modified-SBR enables low biomass yield. Novak et al. (2006) showed that proteins and iron are released in the sidestream and when the biomass from the sidestream is taken to the main bioreactor, the released proteins are degraded along with the organics coming with the influent enabling in low biomass yield. In this research, we showed that the conditions in the sludge minimizing modified-SBR are well-suited, possibly due to different bacterial community structure, such that the modified-SBR shows different substrate partitioning behavior than the control-SBR. In other words, based on stable isotope of carbon-spiked experiments, it can be concluded that the electrons given away by the donor substrate partitioned more towards energy-producing reaction (i.e., higher f^0_e) and less towards cell synthesis reaction (i.e., low f^0_s) where f^0_e and f^0_s represent fractions of electron partitioning to energy and cell synthesis reactions, respectively (Rittmann and McCarty, 2001).

Microbial Community Composition

Based on TRFLP profiles, it appeared that the modified-SBR has greater diversity of ammonia oxidizing bacteria than in the control-SBR. *N. oligotropha* lineage is more commonly found in the municipal WWTP and drinking water systems (Donisi et al., 2002; Wahman et al., 2007). *N. cryotolerans* and *N. marina* are found in extreme low temperature (Karkman et al., 2011) and in saline or marine environments (Ward et al.,

2000), respectively. Although, the modified-SBR has none of these extreme conditions, the in-situ conditions that prevailed in the modified-SBR because of its mode of operation might have induced conditions for *N. cryotolerans* and *N. marina*-related AOBs to exist. The cloning and sequencing results showed that *N. oligatropha* was the dominant AOB in both SBRs, and the modified-SBR has greater diversity of AOBs than in the control-SBR. Because of the limitation during the cloning, the cloning and sequencing results did not show *N. cryotolerans* and *N. marina*-related AOBs. The total *amoA* copy number in both SBRs was similar and the equal copy number explains why both SBRs showed consistently similar NH_4^+-N removals.

The NOB constitute a more phylogenetically diverse group than the AOBs. Two major groups of bacteria, *Nitrospira* and *Nitrobacter* have been known as key players in nitrite oxidation. Two (16S rRNA-based) biomarkers have been used in the study: NTSPA- biomarker specific to *Nitrospira sp.* and NIT3- biomarker specific to *Nitrobacter sp.* The NIT3 primer has one mismatch from *Bradyrhizobium Japonicum*, *Afipia clevelandensis*, *Afipia felis,* and *Rhodopseudomonas palustris*, which are all α-subclass *Proteobacteria* closely related to Nitrobacter (Regan et al., 2002). This result from TRFLP, as well as cloning and sequencing henceforth indicated that either *Nitrobacte*-related NOBs were not present or were not the key players in both SBRs, and demands for further insight on microbial ecology, diversity and ecophysiology on *nitrobacte*r to understand its performance. The present of Nitrospira, supporting the nitrification happened in both reactors. Compared with nitrobacter, *Nitrospira*-like bacteria are widely distributed in different natural and engineered ecosystems (Burrell et al., 1998; Hovanec et al., 1998; Daims et al., 2001). Periodic nitrite accumulation in the

control-SBR was recorded whereas very little or no nitrite accumulation was observed in the modified-SBR. Because, TRFLP and cloning and sequencing revealed the presence of similar NOB communities in both SBRs, nitrite accumulation in the control-SBR was due to some other reason which may be related to the other genus other than *Nitrospira* and *Nitrobacter*.

The difference in PAO ecology observed in control and modified-SBRs may be due the fact that the two different solids retention time (SRT) in the reactors contribute to different ecologies of the "*Candidatus* Accumulibacter"-related PAOs. It was also interesting to note that none of the *ppk1* clones from either reactor were associated with clades IB, IC, IIB, IIC, IID, IIE, and IIF (He et al., 2007; Peterson et al., 2008; He et al., 2010; Kim et al., 2010; Slater et al., 2010), accentuating that the PAOs in the SBRs were highly enriched over a period of two years.

It was interesting to observe a greater diversity of PAOs in the modified-SBR. The modified-SBR was run at 175-days SRT which was much higher than the SRT in the control-SBR. The optimum SRT for EBPR process is typically 5-15 days with 10 days being the most commonly used one (Fukase et al., 1985; Shao et al., 1992; Rodrigo et al., 1996). Several efforts in the past have evaluated the effect of starving conditions on PAOs and have shown that PAOs can use their intracellular polymers, glycogen, and/or polyphosphates as their energy source during starvation period (Miyake and Morgenroth, 2005; Pijuan et al., 2009). However, none of the past efforts evaluated the effect of long SRT or starvation conditions on PAOs ecology. In this study, the recycling of settled biomass to the sidestream reactor in the sludge minimizing modified-SBR perhaps subjected the PAOs to starving conditions in the sidestream reactor followed by feasting

state when the biomass from the sidestream reactor was taken to the modified-SBR. Although, it is not completely proved here, we hypothesize that these alternate starvation (fasting) and feasting conditions have induced conditions which forced this modified-SBR to select for a greater diversity of PAOs. But as Kaewpipat and Grady (2002) found out that replicate activated sludge systems are not identical in microbial population dynamics, it is difficult to conclude the cause of changes in PAOs population in both reactors.

Summary

This research successfully demonstrated that simultaneous nutrient removal and sludge minimization can be achieved sustainably. The strategy of small quantity of sludge wastage in the sludge minimizing reactor helped sustain the modified-SBR and provided a paradigm shift for operational strategies related to sludge minimizing activated sludge processes. The performance of the sludge minimizing reactor was compatible or better than the performance of the control reactor in terms of nutrient and COD removal. The operating conditions in the sludge minimizing reactor provided an ecological niche and this bioreactor showed more diverse PAOs and AOBs ecology than the control bioreactor.

In terms of the biomass yield, the modified-SBR enabled almost 60 % less observed yield than in the control-SBR. Analysis also showed that the mechanisms of sludge minimization in the sludge minimizing bioreactor are more than just endogenous decay, that is, the major mechanism in the conventional anaerobic digestion. Stable isotope-based carbon mass balance revealed that more organic carbon is converted to CO_2 gas in the sludge minimizing bioreactor leading to low biomass yield as compared to

organic matter degradation in the control bioreactor.

This study was performed with synthetic wastewater. Future efforts should focus on running similar reactors but using real primary effluent. The real wastewater contains recalcitrant organics as well and it will be interesting to evaluate how the proposed sludge minimizing operational strategy works in the presence of recalcitrant organic and inert materials. Furthermore, future efforts should also focus on ecophysiology of key microbial community, especially when novel bacteria are encountered. This will ensure the true participation of novel bacteria in the metabolic processes relevant in the reactors.

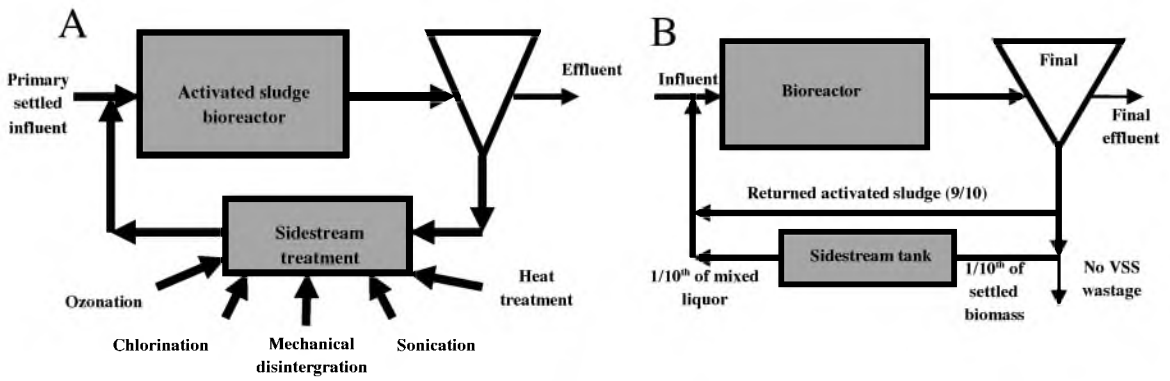

Figure 3.1: Schematic of activated sludge configurations; A. various physical and chemical methods used to achieve biomass reduction; B. schematic of a typical sludge minimizing activated sludge process through returned biomass fasting (in the sidestream tank) and feasting (in the bioreactor).

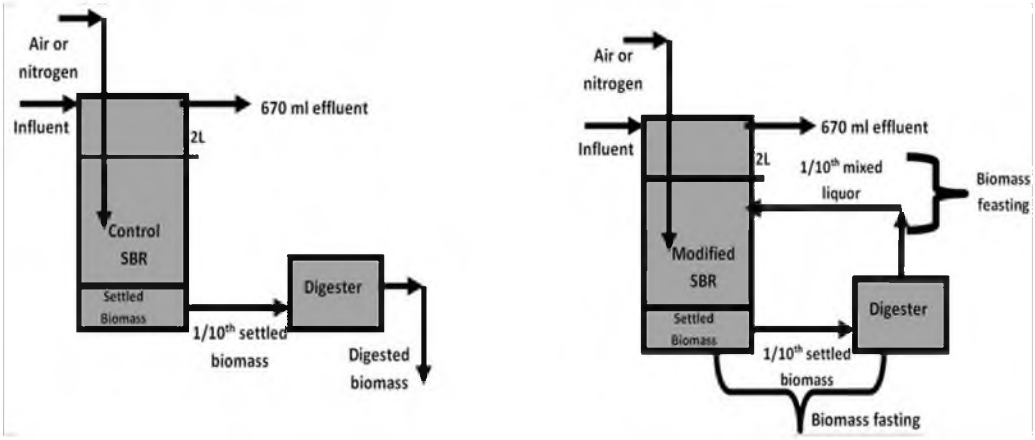

Figure 3.2: Schematics of the control (left side) and the modified (right side) SBRs

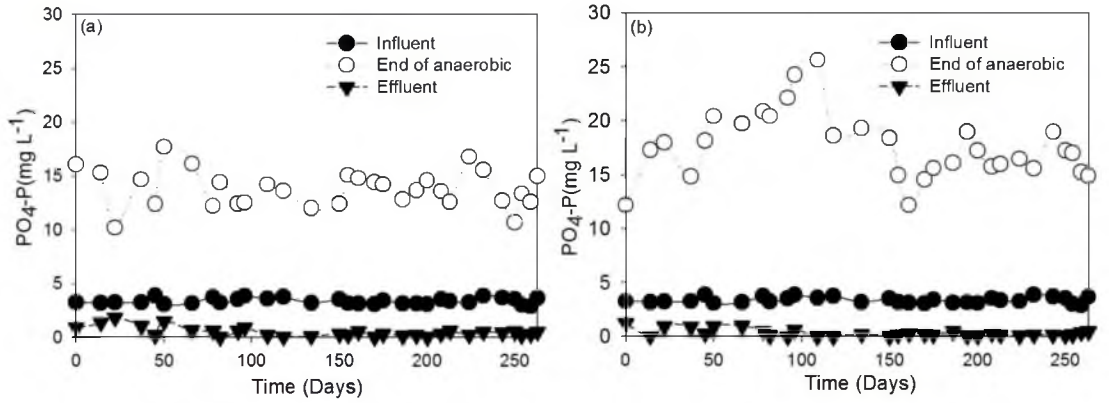

Figure 3.3: Dissolved phosphorus in (a) control-SBR and (b) modified-SBR

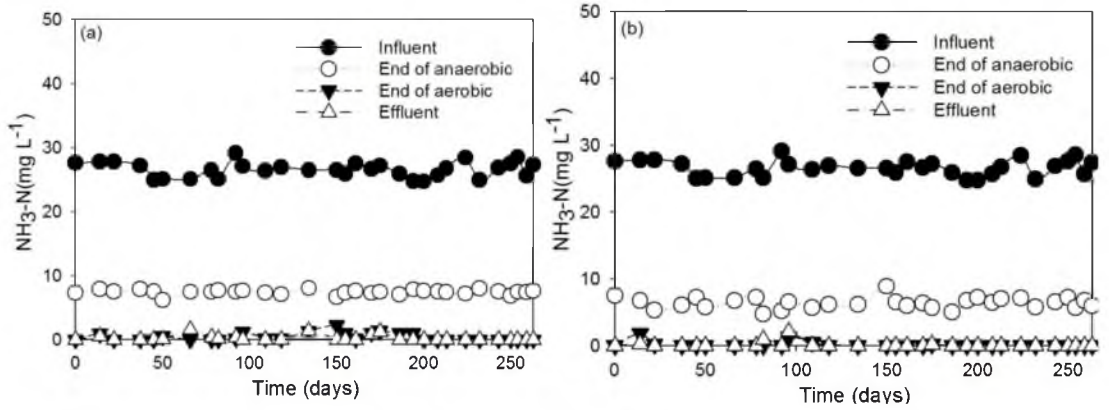

Figure 3.4: Ammonia nitrogen concentrations profiles in (a) control-SBR and (b) modified-SBR

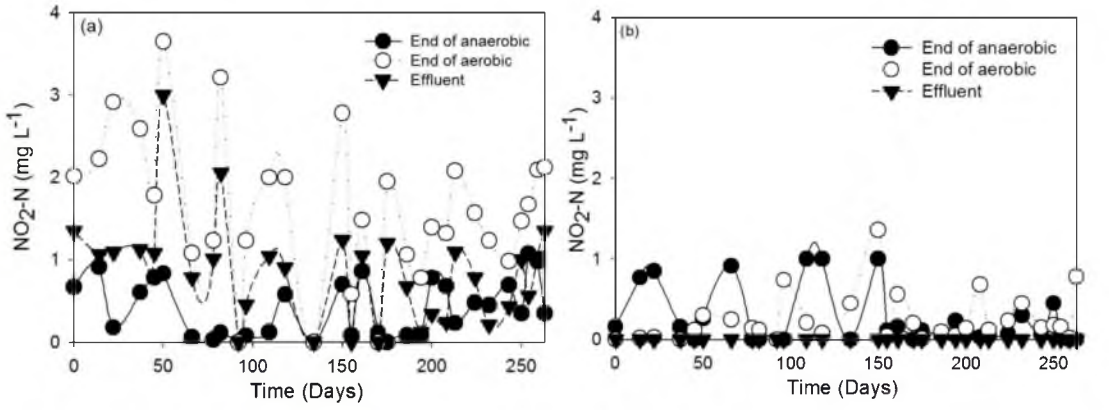

Figure 3.5: NO_2-N concentrations profiles in (a) control and (b) modified SBR

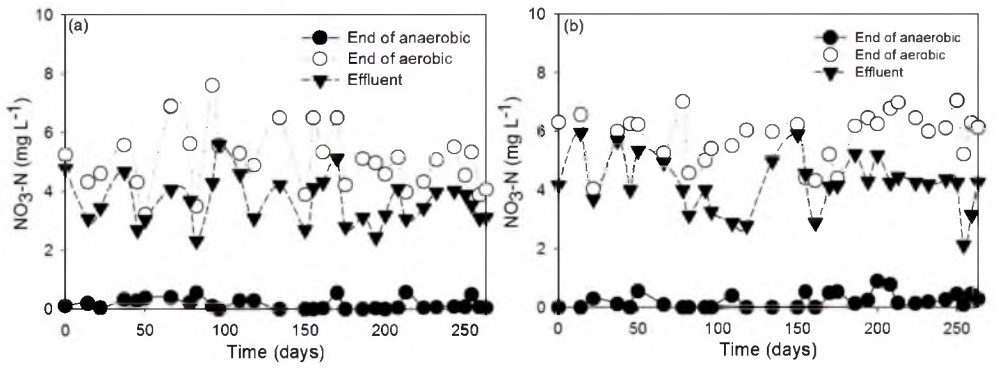

Figure 3.6: NO_3-N concentrations profiles in (a) control and (b) modified-SBR

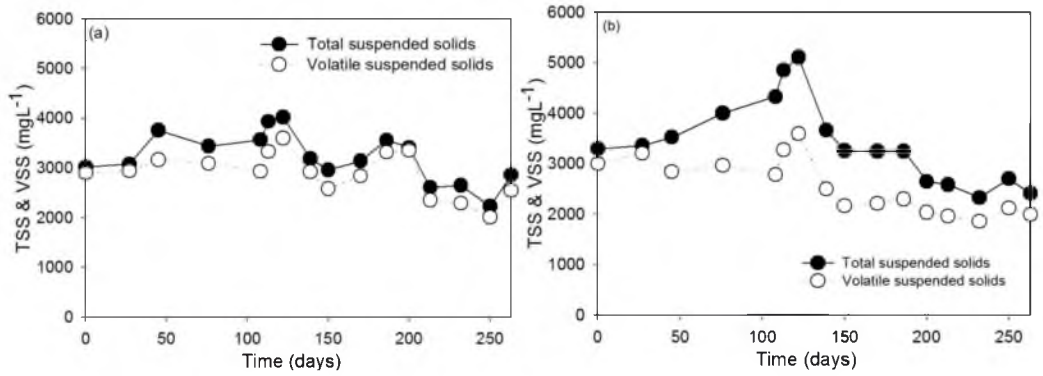

Figure 3.7: Total and volatile solids in (a) control and (b) modified SBR

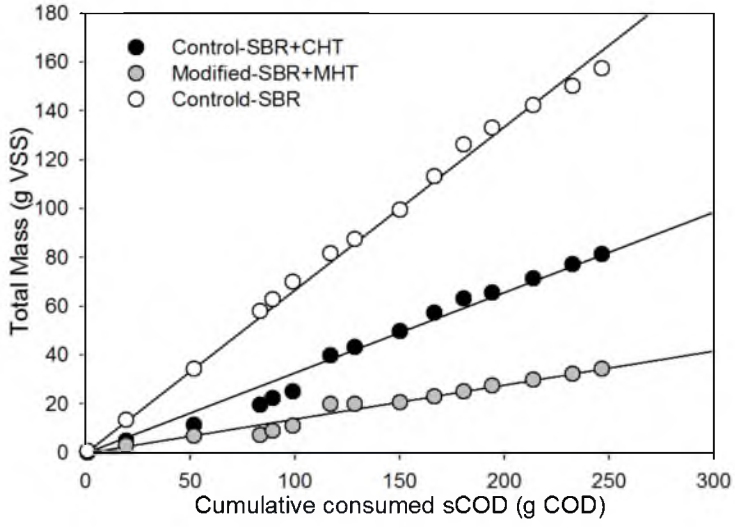

Figure 3.8: The overall yield in the control system, modified system and control-SBR

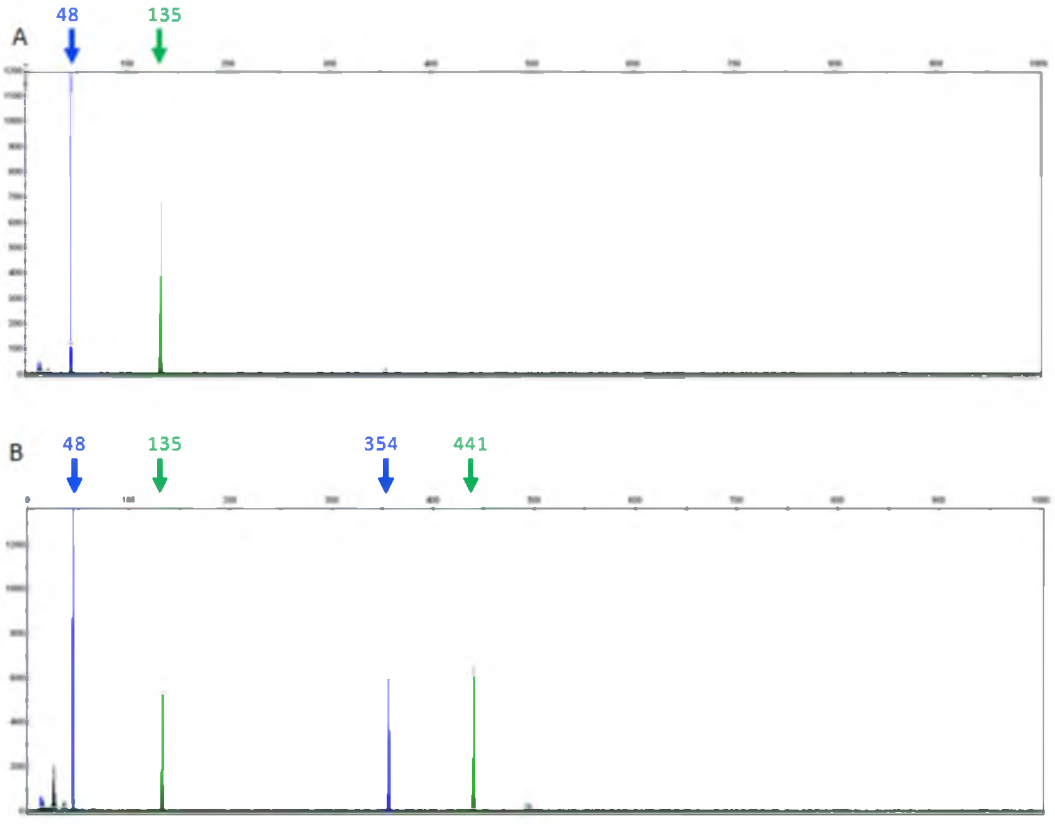

Figure 3.9: Chromatograms representing TF (Terminal Fragments) analysis of the amoA genes obtained from A) Control-SBR and B) Modified-SBR. The x-axes indicate5'-terminal fragment size in base pairs and the y-axes shows fluorescent intensity.

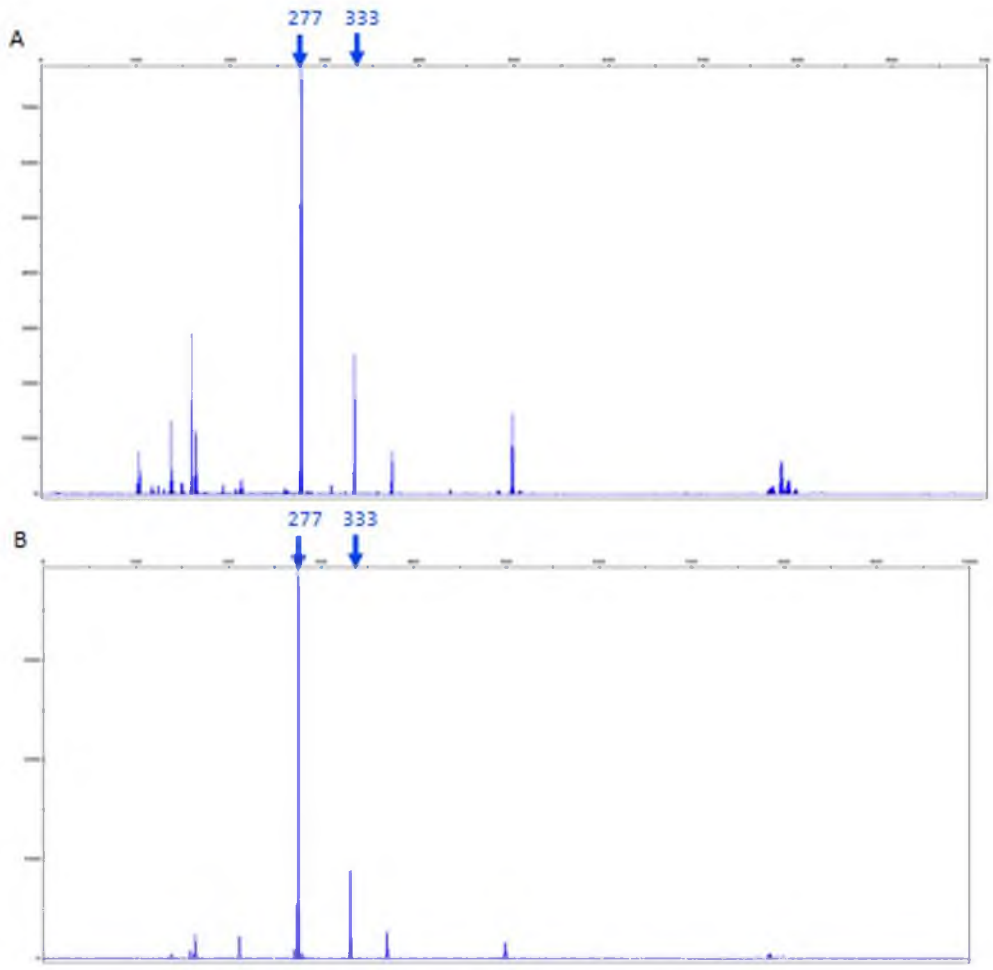

Figure 3.10: Chromatograms representing TF (Terminal Fragments) analysis of the Nitrospira species obtained from A) Control-SBR and B) Modified-SBR. The x-axes indicate5'-terminal fragment size in base pairs and the y-axes shows fluorescent intensity.

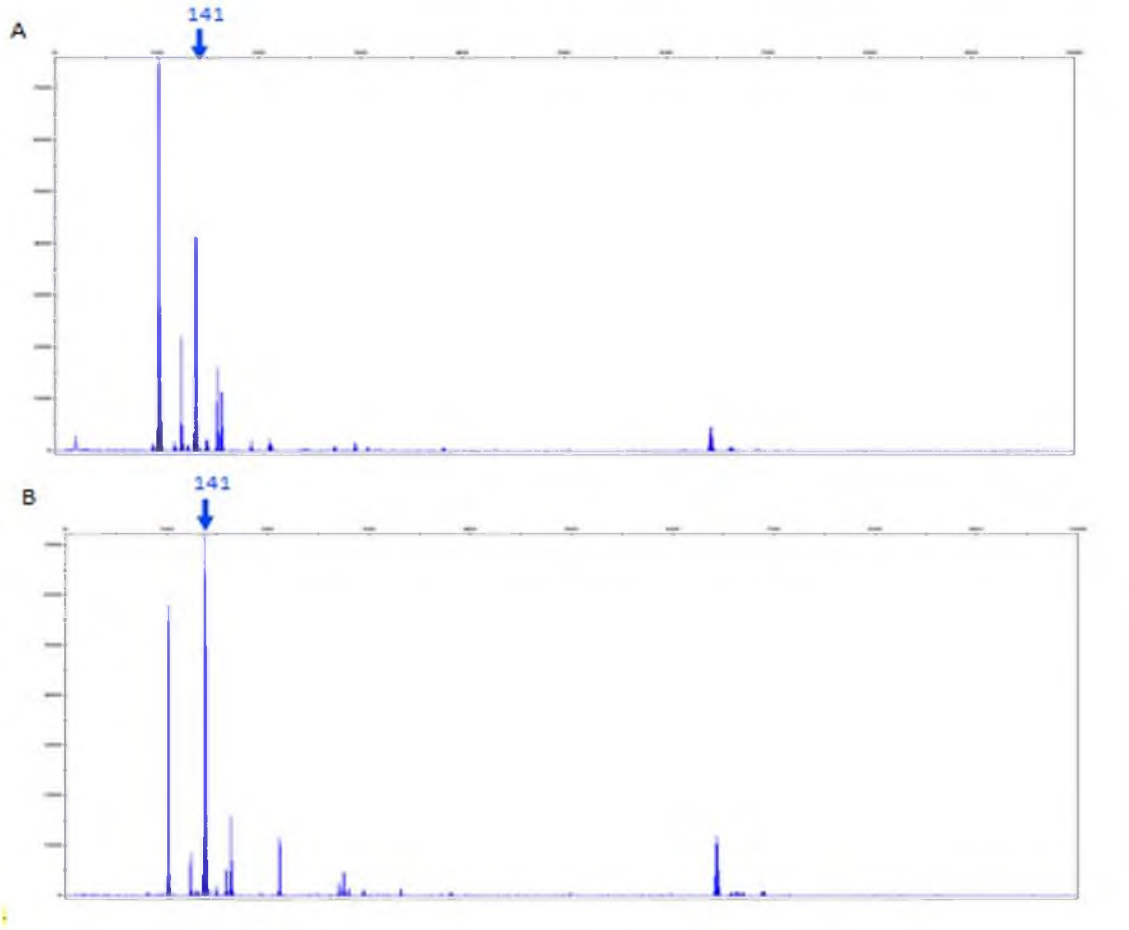

Figure 3.11: Chromatograms representing TF (Terminal Fragments) analysis of the Nitrobacter species obtained from A) Control-SBR and B) Modified-SBR. The x-axes indicate5'-terminal fragment size in base pairs and the y-axes shows fluorescent intensity.

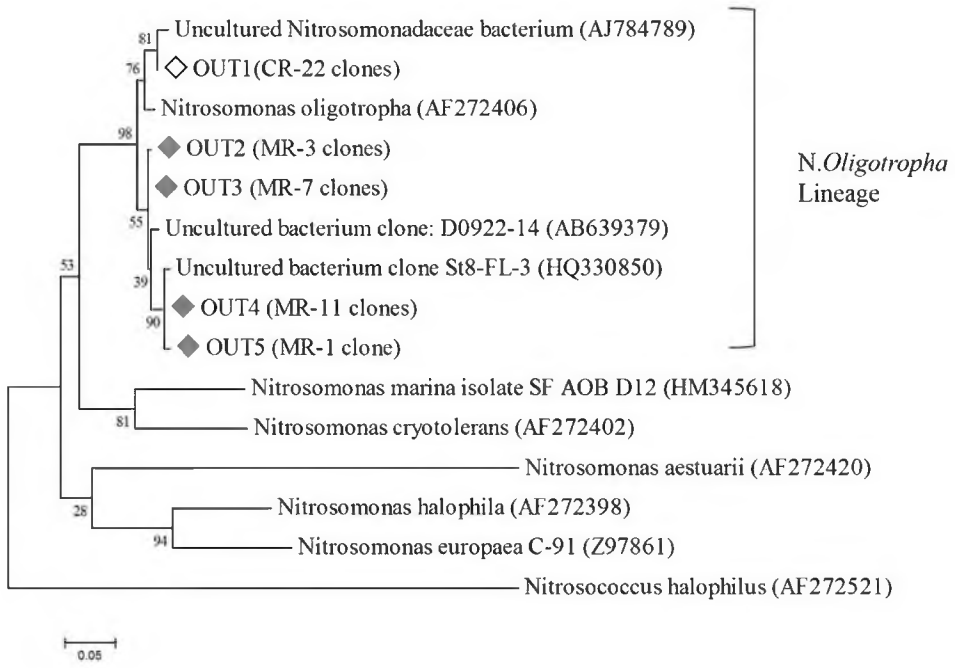

Figure 3.12: Maximum likelihood tree generated from an alignment of amoA gene from both reactors with respect to representative amoA sequences obtained from other studies. The bar represents 0.05 estimated changes per nucleotide.

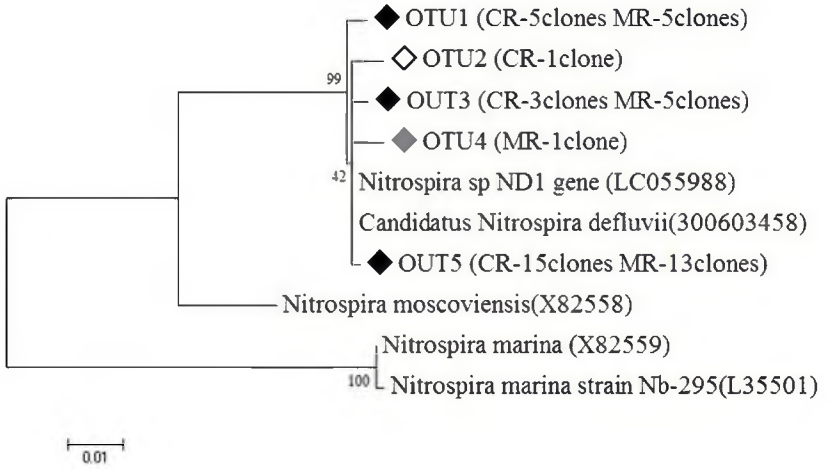

Figure 3.13: Maximum likelihood tree generated from an alignment of 16s rDNA from both reactors with respect to representative Nitrospira 16s sequence obtain from other studies. The bar represents 0.01 estimated changes per nucleotide.

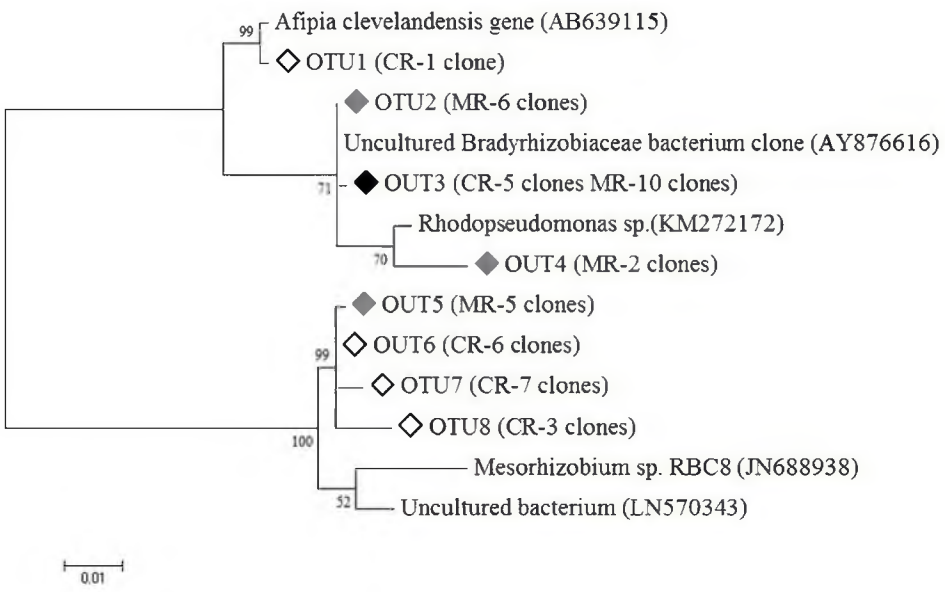

Figure 3.14: Maximum likelihood tree generated from an alignment of 16s rDNA from both reactors with respect to representative Nitrobacter 16s sequence obtain from other studies. The bar represent 0.01 estimated change per nucleotide.

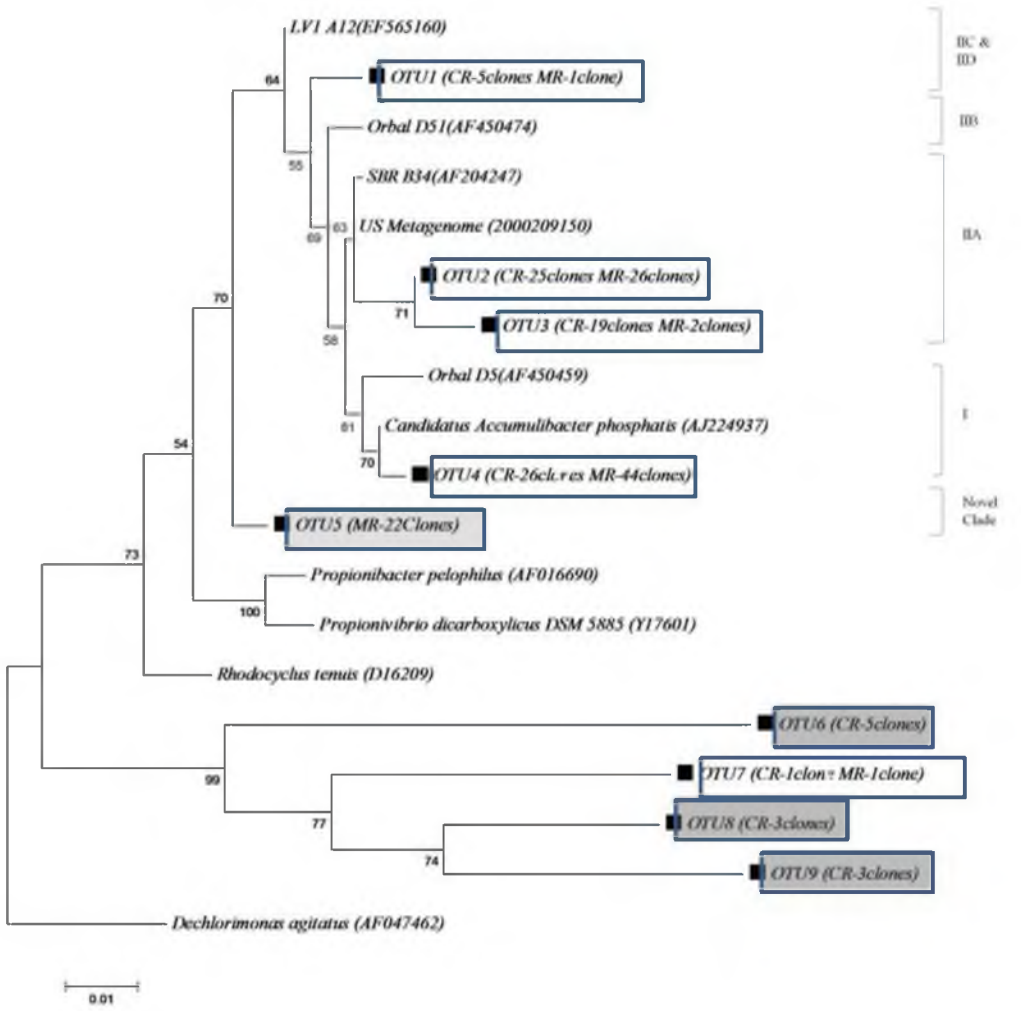

Figure 3.15: Phylogram indicating inferred relatedness of 16S rRNA genes from the "Candidatus Accumulibacter" lineage. Clones common in both SBRs are marked with white rectangles, clones belonging to the control-SBR are marked with gray rectangles and clones belonging to the modified-SBR are marked with light gray rectangles. The bar represents 0.01 estimated changes per nucleotide.

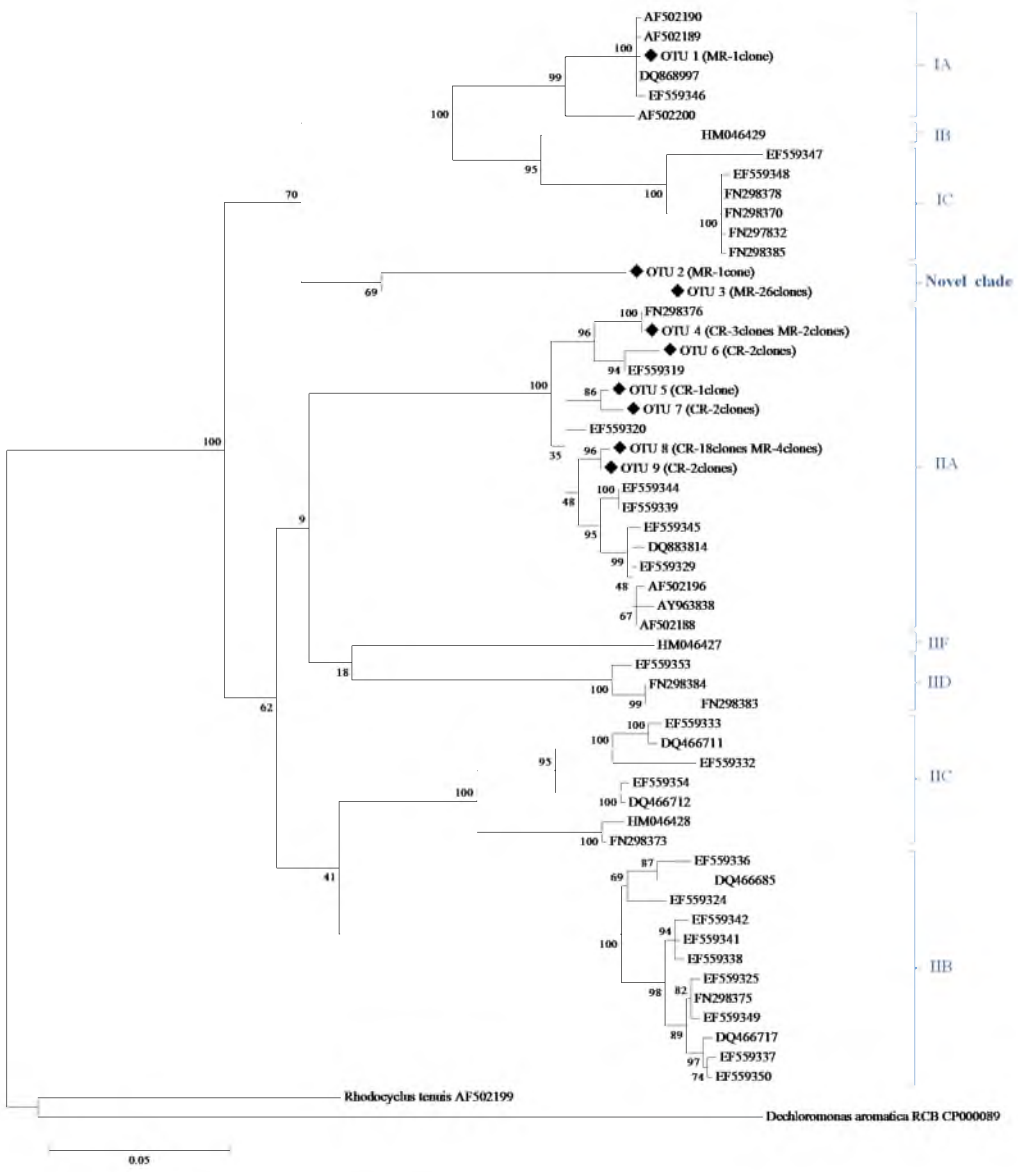

Figure 3.16: Phylogram indicating inferred relatedness of ppk1 genes from the "CandidatusAccumulibacter" lineage. The bar represents 0.05 estimated changes per nucleotide.

COST AND ENERGY COMPARISON OF THE CANNIBAL™ PROCESS AND CONVENTIONAL SLUDGE HANDLING PROCESSES

Four scenarios were assumed for the disposal of biosolids: 1) land application by conventional aerobic digestion to generate Class B biosolids (40 CFR Part 503 Rule), this option was consisting primary clarifier, gravity thickener, aerobic digester, sludge dewatering, and finally land application (Figure 4.1a); 2) Couples with the Cannibal™ process (including mixed liquor fine screen (ML screen)), conventional activated sludge process (CAS) and interchange bioreactor (IBR)) with scenario 1 (Figure 4.1b); 3) landfill, which is incorporated with the primary clarifier, the gravity thickener and the sludge dewatering (Figure 4.1c); and 4) Couples with Cannibal™ process with scenario 3 (Figure 4.1d). From most of the full-scale Cannibal™ process, the primaries were usually eliminated after the Cannibal™ process was introduced. For the aerobic digester, oxygen is supplied either by surface aerators (mechanical aeration) or by diffusers (diffused aeration). In the scenario 3 and 4, the sludge dewatering process includes either centrifuge dewatering or belt filter press dewatering. The design criteria regarding influent wastewater flow was 5MGD, and contained 240mg/L BOD and 200mg/L TSS. Sludge production in scenario 1 and 3 was 0.5lb TSS/BOD, and the primary sludge was 50% of the WAS (Peccia and Westerhoff, 2015). For scenario 2 and 4, the sludge production was 0.2lb TSS/BOD and the sludge removed by ML screen was 0.1lb

TSS/BOD. Based on the EPA Handbook (1984), the sludge mass balance was calculated for each option (shown in Table 4.1 and 4.2).

The total capital cost, operation and maintenance (O&M) cost for primary clarifier were calculated according to the EPA Manual (1980), regarding similar capacity facilities. The total capital cost and O&M cost for all the sludge handling process were followed by the cost estimation method developed in the EPA Handbook (1984). The costs for the Cannibal™ process were adapted from full-scale design data with additional costs from design, contingencies, and interest, while its O&M cost for ML screen and IBR were based on the full-scale application data.

Each treatment option was conducted using a 20-year life and a 3% discount rate. A number of different references were used for cost estimation. All the costs were adjusted for inflation using the Engineering News Record Construction Cost Index (ENRCCI). The costs derived with the algorithms are updated internally using a combination of ENRCCI and the Marshall and Swift Equipment Cost Index (MSECI).

Comparing the costs from scenario 1 and 2 (Table 4.3 a and b, respectively), the O&M net present value (NPV) and energy cost NPV in scenario 2 was much less than in scenario 1, most likely due to cost from the digester, chemical conditioning, and the land application. The life capital costs of these two scenarios were similar when using the mechanical aerobic digester. However, when the mechanical aerobic digester was replaced to diffused aerobic digester, the total capital cost in scenario 1 was 1 million more than scenario 2.

On the other hand, the capital cost of scenario 4, which was combined with the Cannibal™ process, was approximately 20-30% more than scenario 3, when either the

centrifuge or the belt filter press was used for sludge dewatering (Table 4.4a and b). However, the O&M NPV of the scenario associated with Cannibal™ process (Table 4.4 b) was estimated to be 45% less than option 3) because of much lower cost in the chemical conditioning. Although centrifugal dewatering can remove more water and produce a drier "cake," it has high power consumption (EPA, 2000). By using centrifuge, the energy cost NPV was evaluated as twice as much when using belt press and option 4) (Table 4.4).

In summary, the Cannibal™ sludge minimization process can be incorporated with new or existing wastewater treatment plants to reduce the O&M and/or capital costs.

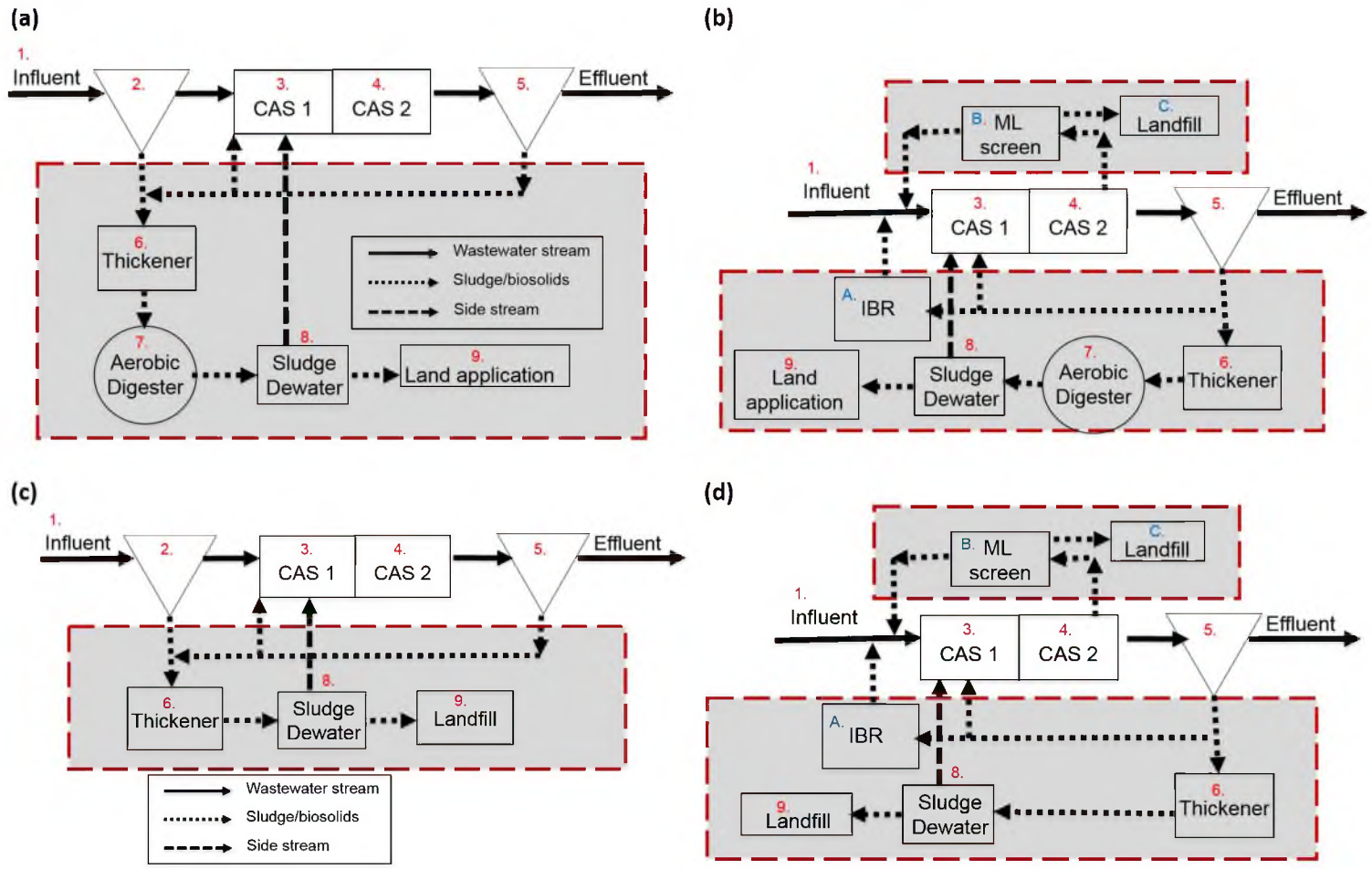

Figure 4.1: Flow schematic of the four scenarios to dispose sludge: scenario 1) (a), scenario 2) (b), scenario 3) (c), scenario 4) (d).

Table 4.1: Sludge mass balance summary in scenario 1 (a) and 2 (b).

(a)

Location	Description (conventional)	calculated average solids	calculated average volume	calculated average volume	Estimated average solids concentration
		DSS (lb/day)	SV (gal/day)	SV (MG/Y)	SS (%)
2	Primary sludge	5,004	30,000	10.95	2.00%
5	WAS	3,545	85,000	31.03	0.50%
6	Total (primary and WAS)			41.98	0.89%
7	thickened sludge	7693.65	26250.00	9.58	3.60%
7	total mass to digester (solids+water)	221,114			
	mass output	215,729			
8	remained solids	5,386			
	sumpernatant reture	345	13793	5.03	
8	digested sludge withdrawal	5040	11968	4.37	5.00%
	solids destroyed in digester	2308	-	-	-
8	chemically conditioned sludge	5053	11998	4.38	5.00%
	use polymers (5lb/ton)	13			
8	centrate return	404	9048	3.30	0.54% (5400mg/L)
9	dewatered sludge	4649	2949	1.08	18.00%

(b)

Location	Description (with Cannibal® process)	calculated average solids	calculated average volume	calculated average volume	Estimated average solids concentration
		DSS (lb/day)	SV (gal/day)	SV (MG/Y)	SS (%)
2	inert	1,001	24,000	8.76	2.00%
8	screened inert	901	360	0.13	30.00%
5	WAS	2,002	48,000	17.52	0.50%
6	thickened WAS	1,801	7,200	2.63	3.00%
7	total mass to digester (solids+water)	60,648			
	mass output	59,658			
8	remained solids	991			
	sumpernatant reture	127	5088	1.86	
8	digested sludge withdrawal	864	2050	0.75	5.00%
	solids destroyed in digester	811	-	-	-
8	chemically conditioned sludge	866	2050	0.75	5.00%
	use polymers (5lb/ton)	2			
8	dewatering centrate return	69	1545	0.56	0.54% (5400mg/L)
9	dewatered sludge	796	505	0.18	18.00%

Table 4.2: Sludge mass balance summary in scenario 3 (a) and 4 (b).

(a)

Location	Description (conventional)	calculated average solids	calculated average volume	calculated average volume	Estimated average solids concentration
		DSS (lb/day)	SV (gal/day)	SV (MG/Y)	SS (%)
2	Primary sludge	5,004	30,000	10.95	2.00%
5	WAS	3,545	85,000	31.03	0.50%
6	Total			41.98	0.89%
	thickened sludge	7693.65	26250.00	9.58	3.60%
8	chemically conditioned sludge	7732	26250	9.58	4.00%
	use polymers (10lb/ton)	38			
8	dewatering centrate return	619	21737	7.93	0.34% (3400mg/L)
9	dewatered sludge	7114	4513	1.65	18.00%

(b)

Location	Description (with Cannibal® process)	calculated average solids	calculated average volume	calculated average volume	Estimated average solids concentration
		DSS (lb/day)	SV (gal/day)	SV (MG/Y)	SS (%)
2	inert	1,001	24,000	8.76	2.00%
B	screened inert	901	360	0.13	30.00%
5	WAS	2,002	48,000	17.52	0.50%
6	thickened WAS	1,801	7,200	2.63	3.00%
8	chemically conditioned sludge	1810	7200	2.63	4.00%
	use polymers (10lb/ton)	9			
8	dewatering centrate return	145	6143	2.24	0.28% (2800mg/L)
9	dewatered sludge	1666	1057	0.39	18.00%

Table 4.3: Life cycle cost summary in scenario 1 (a) and 2 (b)

(a)

20 years 5MGD Life Cycle Cost		Capital Cost	O&M NPV	Energy Cost NPV
Primary clarifier		675,811	107,346	17,727
Gravity Thickening		849,004	1,023,561	19,499
Aerobic Digester	Mechanical	1,010,719	2,887,878	886,335
	Diffused	2,425,725	3,460,292	1,772,670
Chemical Conditioning	Polymers	141,501	1,890,902	
Dewatering	Belt press	1,010,719	971,229	35,453
Land Application		363,859	1,018,246	
Total	**With Aerobic Digester (mechanical)**	**4,051,612**	**7,899,161**	**959,014**
	With Aerobic Digester (diffused)	**5,466,618**	**8,471,575**	**1,845,349**

(b)

20 years 5MGD Life Cycle Cost (with Cannibal® process)		Capital Cost	O&M NPV	Energy Cost NPV
Cannibal ®	ML screen	1,848,000	148,869	40,771
	landfill		339,688	
	IBR		668,296	19,499
Gravity Thickening		606,431	996,767	20,373
Aerobic Digester	Mechanical	687,289	1,386,268	177,267
	Diffused	1,212,863	1,787,259	354,534
Chemical Conditioning	Polymers	1,026,489		
Dewatering	Belt press	706,508	706,508	17,727
Land Application		40,429	227,294	
Total	**With Aerobic Digester (mechanical)**	**4,915,146**	**4,473,691**	**275,638**
	With Aerobic Digester (diffused)	**5,440,720**	**4,874,682**	**452,905**

Table 4.4: Life cycle cost summary in in scenario 3 (a) and 4 (b)

(a)

20 years 5MGD Life Cycle Cost		Capital Cost	O&M NPV	Energy Cost NPV
Primary clarifier		675,811	107,346	17,727
Gravity Thickening		808,575	1,023,561	19,499
Chemical conditioning	Polymers	202,144	3,268,884	
Dewatering	Centrifuge	1,212,863	1,727,765	531,801
Landfill			3,396,878	
Total		**2,899,392**	**9,524,435**	**569,027**
Primary clarifier		675,811	107,346	17,727
Gravity Thickening		808,575	1,023,561	19,499
Chemical Conditioning	Polymers	202,144	3,268,884	
Dewatering	Belt press	1,010,719	1,742,345	70,907
Landfill			3,396,878	
Total		**2,697,248**	**9,539,014**	**108,133**

(b)

20 years 5MGD Life Cycle Cost (with Cannibal® process)		Capital Cost	O&M NPV	Energy Cost NPV
Cannibal®	ML screen	1,848,000	148,869	40,771
	Landfill		339,688	
	IBR		668,296	19,499
Gravity Thickening		606,431	996,767	20,373
Chemical conditioning	Polymers	129,372	1,119,197	
Dewatering	Centrifuge	889,433	1,144,123	177,267
Landfill			905,834	
Total		**3,473,236**	**5,322,774**	**257,911**
Cannibal®	ML screen	1,848,000	148,869	40,771
	Landfill		339,688	
	IBR		668,296	19,499
Gravity Thickening		606,431	996,767	20,373
Chemical Conditioning	Polymers	129,372	1,119,197	
Dewatering	Belt press	1,010,719	952,123	35,453
Landfill			905,834	
Total		**3,594,522**	**5,130,774**	**116,097**

RESPONSE OF LABSCALE SIMUTANEOUS BNR AND SLUDGE MINIMIZATION REACTOR WHEN THE OPERATION IS CHANGED TO REAL WASTEWATER[**]

Abstract

Activated sludge process (ASP) is the most widely used treatment method for municipal wastewater. However, excess biomass generated during the process is one of the main drawbacks. Earlier studies demonstrated nutrient removal from synthetic wastewater using the activated sludge process running in biomass fasting and feasting mode, while simultaneously minimizing biomass production. In this study, we report findings from a lab -scale sludge minimizing BNR reactor, when its operation was changed from synthetic to real wastewater. Two lab -scale sequencing batch reactors; one in sludge minimization (hereafter called modified-SBR) and the other in conventional activated sludge (referred as control-SBR) modes were operated for more than 300 days. Both reactors were started and operated with synthetic feed. However, the feed to both reactors was changed to 100% real primary effluent collected from a local full-scale wastewater treatment plant in a stepwise manner. Irrespective of the feed composition, more than 98% NH_4^+-N removal was recorded in both SBRs. However, the

[**] This chapter has been adapted and published as a journal paper and can be cited as:
Huang, P., Goel, R., 2015. Response of a sludge minimizing lab scale BNR reactor when the operation is changed to real wastewater. Water Res. 81, 301-310.

total dissolved phosphorus removal decreased from an overall 89% at 100% synthetic feed to nearly 80% at 100% real primary effluent in both SBRs. The overall observed sludge reduction in the modified-SBR as compared to the sludge yield in the control-SBR also decreased from 65% to 39%, when the feed was changed from 100% synthetic to 100% primary effluent. Finally, both SBRs were fed with the raw wastewater (after it was screened) from another wastewater treatment plant for approximately 100 days. Both reactors achieved more than 95% NH_4^+-N removal and 80% dissolved phosphorus removal. The overall observed sludge yield in the modified-SBR was 35% lower than in control-SBR. The phosphorus mass balance was conducted, when the reactors were fed with primary effluent and raw wastewater with approximately 18% of the phosphorus unaccounted for.

Introduction

The activated sludge process (ASP) has been widely used all over the world, since 1930 (Benidickson and Jamie, 2011) for the treatment of municipal and industrial sewage (Grady et al., 1999; Metcalf and Eddy, 1994). ASP can be optimized for effective removal of nitrogen, phosphorus, organic matters, and suspended solids (Grady et al., 1999; Metcalf and Eddy, 1994). Different contaminants present in liquid waste serve as carbon, nitrogen and energy sources for the bacterial community present to grow in ASP. As a result, the bacteria grow and multiply. The treated liquid waste flows to the gravity secondary clarifier where it is allowed to settle. A portion of the settled biomass in the secondary clarifier, called waste activated sludge (WAS), is routinely removed from the bottom of the secondary clarifier and the remaining biomass is recycled back to the bioreactor to maintain a healthy population of bacteria in the bioreactor.

Approximately 8.2 million tons of activated sludge is generated each year in the United States (USEPA, 1999, Wang et al., 2012). In order to meet the requirement of EPA's 40 CFR Part 503 Rule, further treatment of sludge is needed. The treatment of excess sludge is labor and energy intensive and may consumer as much as 65% of the plant's operation budget (Saby et. al., 2003; Chen et al., 2001; Camacho et al., 2002; Cui and Jahng, 2004; Barjenbruch and Kopplow, 2003). Anaerobic and aerobic digestions are the most common posttreatments of sludge that can reduce excess sludge by 40-50%. However they are capital intensive, process-wise complex and need external chemical dosing (Khursheed and Kazmi, 2011). The option for the use of biosolids includes its composting followed by its land application. However, land application of the biosolids is restricted in many states due to potential health risks to people and livestock due to the presence of trace elements (Basta et al., 2005), organic chemicals (Overcash et al., 2005; Xia et al., 2005), pathogens (Gerba and Smith Jr., 2005), odors (Schiffman and Williams, 2005), and nutrients (Pierzynski and Gehl, 2005; Cabrera et al., 2005) in the digested sludge. Handling and disposal of excess sludge is more challenging in coastal areas such as in Florida and California due to the limited and depleting landfill resources. With increasing urbanization and industrialization, the excess sludge will pose increasing challenges. Therefore, sludge reduction at the source becomes an attractive solution to solve sludge-associated problems.

For sludge reduction at the source, a number of technologies have been developed which include lysis-cryptic methods (He and Wei, 2010; Ma et al., 2012; Wang et al., 2011), sludge reduction, based on uncoupling metabolism (Feng et al., 2012; Tang et al., 2011; Xing et al., 2008), and worms' predation (Lou et al., 2011; Tia and Lu, 2010).

Böhler and Siegrist (2006) concluded that all physical, chemical, and thermal processes are expensive and will increase the overall energy consumption of a plant. Guo et al., (2013) reviewed all technologies discussed above, and concluded that sludge reduction through returned sludge fasting and feasting has many more obvious positive effects than other technologies. In general in ASP's accomplishing sludge reduction through returned biomass fasting and feasting, a portion of the returned biomass is taken to an anaerobic side stream tank and an equal portion of the mixed liquor from this side stream tank is recycled back to the main reactor (Figure 5.1). Recycling of returned waste-activated sludge in between the anaerobic tank (i.e., fasting conditions) and the main bioreactor (i.e., feasting conditions) induced conditions which enable a net sludge reduction.

In the past, the nutrient removal component was not addressed in ASP achieving a net sludge reduction at source. However, more recently a few studies (Goel and Noguera 2006, Datta et al., 2009; Huang et al., 2014) have demonstrated that nutrient removal can be coupled with biomass reduction, using a fasting and feasting approach for sustainable wastewater treatment and biomass managemen,t albeit with synthetic wastewater. The use of synthetic wastewater provides ideal conditions but does not represent the complexity of substrate that is present in the real wastewater. For example, the presence of inert and/or recalcitrant COD can directly influence sludge production. In this study, therefore, we aimed to evaluate the operation of simultaneous sludge minimization coupled with nutrient removal by running laboratory-scale sequencing batch reactors (SBRs) with 100 % real wastewater. A more fundamental question that we posed was how an ongoing sludge minimizing bioreactor will respond, when the operation of this reactor will be slowly changed from synthetic to real wastewater. On the other hand, the

fate of the phosphorus as P mass balance in this system remained unclear from the previous studies (Goel and Noguera 2006, Johnson et al., 2007). To accomplish these goals, the primary effluent was collected weekly from a local wastewater treatment plant (WWTP) to feed the lab scale reactors. After the reactor performed consistently in terms of nutrients removal, the influent was then changed to raw influent (after it was screened) from the other WWTP. The P mass balance was also conducted when the reactor was fed with the real wastewater.

Materials and Methods

Reactor Operation and Complete Cycle Monitoring

Two 2-L bench-scale SBRs were operated to achieve simultaneous ammonia and phosphorous removal (Figure 5.2). One of these SBRs was operated in the conventional mode at a 10-days SRT and was designated as the control-SBR. The other SBR (called modified-SBR) was operated in sludge reduction mode. The time sequence in each cycle of both SBRs included a 1.5 h anaerobic phase, following followed by a 2.5 h aerobic phase, a 1.5 h anoxic time period and a 0.5 h settling period. Stage I was a 63 days period, when both SBRs were fed with synthetic wastewater. In stage II, both SBRs were fed with a mixture of synthetic and real wastewater in a ratio of 25/75 (v/v) (from day 64 to day 85) and 50/50 (v/v) (from day 86 to day 114). In stage III, both reactors were fed with 100% real primary effluent (from day 115 to day 315) collected from Central Valley Water Reclamation Facility (CVWRF, Utah). Both SBRs were fed with 100% real raw wastewater for 102 days (stage IV) obtained from Snyderviville Basin Water Reclamation District (SBWRD, Utah). The details of these two SBRs and how biomass fasting and feasting was introduced in the modified-SBR are provided elsewhere (Huang

et al., 2014). The SRT of modified system in stage III and IV were operated as 84-days and 70-days, respectively. For yield calculation purpose, the cumulative wastage in terms of sampling wastage solids present in the final effluent was also considered.

Analytical Methods and Statistical Analysis

Samples were routinely collected at the end of each phase, filtered at 0.45μm and analyzed. Chemical oxygen demand (COD) and ammonium (NH_4^+-N) were quantified, using HACH methods 8000 and 10031 (Salicylate method), respectively. Nitrate (NO_3^--N), nitrite (NO_2^--N), and dissolved phosphorus (PO_4^{3-}P) were determined by using EPA method 300.0. Readily biodegradable COD was measured with physical-chemical methods described in Mamais et al. (1993). Total phosphorus (TP) in the wastewater and in the mixed liquor was quantified using HACH method 10127 and standard method 4500 PE (perchloric acid digestion) (APHA, 1985), respectively. Mixed liquor samples were collected at mid-height of the bioreactors, effluent containers or holding tanks. The mixed liquor solids concentration was determined as total suspended solids (TSS) and as volatile suspended solids (VSS). Both were measured in accordance with standard methods (APHA, 1985).

The Shapiro-Wilk test was used to test for the normality of data sets (Shapiro and Wilk, 1965). When the data was normally distributed, the unpaired, two-tailed student's t-test (Barbara, 2008) was used to identify statistical differences between samples from the control and modified-SBR. If the results were not a normal distribution, the Mann-Whitney U test (Mann and Whitney, 1947) was applied as a nonparametric statistical test. All statistical analyses were performed using the vegan 1.13 within the R software package (Oksanen et al., 2008).

Results

Reactor Performance in Terms of Nutrient Removal

Nutrients removal during stage I. Both reactors were consistently achieving more than 89% of dissolved P removal, more than 99% of NH_4^+-N removal and about 80% of the total inorganic nitrogen (TIN) removal, during stage I. Figure 5.3 (stage I) shows reactor performances in terms of dissolved phosphorus removal in the control-SBR (a) and the modified-SBR (b), including dissolved phosphorus in the influent, effluent, and at the end of the anaerobic phase. The average dissolved PO_4^{3-}P released at the end of the anaerobic phase was higher in the modified-SBR (16.5±1.21mgL^{-1} PO_4^{3-}P), than in the control-SBR (13.7±1.77mgL^{-1} PO_4^{3-}P) ($P<0.05$). The effluent PO_4^{3-}P concentration was lower than 0.50mgL^{-1} in both SBRs. At the end of the anaerobic period, the concentration of COD in both SBRs was below the detection limit (<2mgL^{-1}) and complete COD removal was observed, during most of this period.

Figure 5.3c and d (stage I) shows reactor performance of NH_4^+-N removal. NH_4^+-N concentrations in the effluent from both SBRs were always below detection limit (<2mgL^{-1}), during stage I. The NO_2^--N concentration fluctuated in the control-SBR, while it remained below 0.1 mgL^{-1} in the modified-SBR, at the end of each aerobic cycle. In general the rise in NO_2^--N concentrations in the mixed liquor in the control-SBR corresponded to drops in NO_3^--N concentrations (Figure 5.3e and g). Despite the same influent feeding to both SBRs, NO_2^--N, concentration in the effluent from the modified-SBR was lower than that from the control-SBR ($P<0.05$). The average NO_3^--N concentration at the end of the aerobic cycle in the control-SBR (5.04±1.40 mgL^{-1}) was lower than the NO_3^--N concentration in the modified-SBR (6.02 ± 0.95mgL^{-1}) ($P<0.05$).

Nutrients removal during stage II and III. In stage II the feed to both SBRs was changed to 25% real and 75% synthetic from day 64 to day 85 and to 50% real and 50% synthetic from day 86 to day 114. In stage III, both reactors received 100% real wastewater. During these changes, both reactors achieved above 80% PO_4^{3-}-P, 98% NH_4^+-N removal and 70% TIN removal. Phosphorus concentration in the control-SBR decreased (Figure 5.3a), when the influent was changed to real wastewater in 3 steps. The PO_4^{3-}-P released at the end of the anaerobic phase also decreased to 11.3±0.41mgL^{-1} with 25% real wastewater, 6.9±0.52mgL^{-1} with 50% real wastewater and then, finally, to 4.8±0.47mgL^{-1} with 100% real wastewater. The same trend was observed in the modified-SBR. The released PO_4^{3-}-P concentration of 16.5 mgL^{-1} was recorded during stage I and it decreased to 14.69±3.55mgL^{-1} with 25% real wastewater, then to 12.94±2.35mgL^{-1} with 50% real wastewater, and, finally, to 6.98±1.59mgL^{-1}. However, the released P was always higher in the modified-SBR than in the control-SBR, during all stages ($P<0.05$). The effluent PO_4^{3-}-P concentration was consistently lower than 0.6mgL^{-1} in both SBRs during stage II and stage III. The overall COD removal efficiency in both reactors was above 80% all the times.

As shown in panels c and d of Figure 5.3, the NH^+_4-N removal efficiencies were unaffected due to feed changes and the NH^+_4-N concentrations in the final effluent of both SBR always remained below detection limits. Surprisingly, the NO_2^--N concentrations at the end of aerobic period and in the effluent during phase II in the control-SBR went as high as 7 mgL^{-1} with a steady drop thereafter. With 50% real wastewater, NO_2^--N concentrations at the end of aerobic phase increased about 3 fold, then decreased and finally became steady at 0.37±0.32 mgL^{-1} with 100% wastewater. The

relatively high NO_2^--N concentrations at the end of the aerobic time period in the control-SBR suggested incomplete nitrification causing accumulation of NO_2^-. The rises and drops in NO_2^--N concentrations in control-SBRs corresponded to drops and rises in NO_3^--N concentrations (Figure 5.3e and g). Furthermore, it is also significant that for the control-SBR, the NO_2^--N concentrations in the final effluent are intermittently higher than those at the end of the aerobic phases. This could have been due to partial denitrification of nitrate to nitrite, during the last anoxic phase. However, except on a few occasions, during phase II, the NO_2^--N concentrations in the modified-SBR were always below 0.1 mg $L^{-1,}$ indicating complete nitrification. The average NO_3^--N concentrations were 7.42 ± 1.49 and 7.97 ± 1.45mg L^{-1} in the control and modified-SBR, respectively (Figure 5.3g and h). Furthermore, the difference in NO_3^-N concentrations at the end of aerobic period (open white circles) and the last anoxic period (i.e., effluent) (inverse black triangles) demonstrate active denitrification.

Nutrient removal during stage IV. Compared with the primary effluent that was used in stage III, the raw wastewater contained higher phosphorus concentrations (4.85 ± 1.22 mgL^{-1}). Figure 5.4a and b (stage IV) shows reactor performance in terms of dissolved phosphorus. The released P in the end of anaerobic period was 1.22mgL^{-1} higher in modified-SBR than in the control-SBR, during stages IV ($P<0.05$). The effluent PO_4^{3-}-P concentration was lower than 0.8mgL^{-1} in both SBRs, during this stage, except on days 16, 28, 30, and 52 in the modified-SBR. The high PO_4^{3-}-P effluent concentration on days 16 and 52 was due to the fact that the higher simulated PO_4^{3-}-P concentration (over 50 mgL^{-1}) recycled back to the mainstream modified-SBR contributed to the >1.25 mgL^{-1} higher concentration of PO_4^{3-}-P in the beginning of the cycle. The effluent PO_4^{3-}-P

went back to 0.5mg/L after a few cycles, by replacing the supernatant from the sidestream reactor attached modified-SBR with DI water during these two dates. The higher effluent PO_4^{3-}-P concentration on other dates might be due to the readily biodegradable COD (rbCOD) deficiency. The overall COD removal efficiency in modified-SBR was 82%, which was higher than that in control SBR (78%, $P<0.05$).

The average NH^+_4-N concentration in the raw wastewater from SBWRD of 32.5 ± 6.72 mgL^{-1}, was higher and more fluctuating than that in the primary effluent from CVWRF. The change of feed initially affected NH^+_4-N removal efficiency in the control-SBR for the first couple of days, resulting in higher than 6mg/L NH^+_4-N in the effluent (Figure 5.4 c). Overall, the removal efficiency of NH^+_4-N was 95% and 98% in the control-SBR and the modified-SBR, respectively. As soon as the influent changed to raw wastewater, higher NO_2-N (2.95±1.85mgL^{-1}) levels and lower NO_3-N (5.88±1.60mgL^{-1}) levels were again observed in the control-SBR, than in the modified-SBR after the aeration period, suggesting the incomplete nitrification in the control-SBR. The NO_2-N and NO_3-N in modified-SBR were 0.72±0.86mgL^{-1} and 10.2±2.66mgL^{-1} in the same period, respectively (Figure 5.4f and h). However, the higher NO_2-N (>2 mgL^{-1}) concentration in the effluent after day 30 in the modified-SBR was determined, during this stage. The decrease in NO_3^--N concentration and increase of NO_2^--N, during the anoxic period indicated denitrification in both SBRs (Figure 5.4e - h).

Solids and Biomass Yield

TSS and VSS in both SBRs. According to the Figure 5.5a, the average TSS and VSS concentrations were constant in the control-SBR throughout the study. In contrast, fluctuations in TSS and VSS in the modified-SBR were observed during stage I. When

the feed to the modified-SBR was mixed with 25% and later 50 % primary effluent, during stage II, TSS and VSS increased to 5800mgL^{-1} and 4500mgL^{-1}, respectively. At this point, a known volume of the settled biomass from the modified-SBR was pumped to the attached sidestream reactor to control the solids buildup in the modified SBR reactor. Following this change, the operation of the reactor was switched to the feed containing 100% primary effluent. The solids concentrations in the modified-SBR decreased and stabilized at 2430 mgL^{-1} after day 217 in stage III. The sludge concentration during stage IV in both SBRs became more stable. Figure 5.6a and b show TSS and VSS in the control-SBR and the modified-SBR in stage IV, respectively.

The average TSS and VSS concentrations in the anaerobic digester associated with the control-SBR were 3230±746 mgL^{-1} and 2920±689 mgL^{-1}, respectively, during stage I, and were 4031±504 mgL^{-1} and 3453±474 mgL^{-1}, 2603±455 mgL^{-1}, and 2153±382 mgL^{-1} during stage III and IV, respectively (figure not included). On the other hand, the average TSS and VSS concentrations in the sidestream reactor attached to the modified-SBR were 3860±493 mgL^{-1} and 2910±843 mgL^{-1}, respectively, and these numbers were 4333±657 mgL^{-1} and 3215±547 mgL^{-1}, 2747±361 mgL^{-1} and 1974±610 mgL^{-1} during stage III and IV.

Observed biomass yields in both SBRs. The observed biomass yield for the control system (control-SBR and the attached digester) and the modified system (modified-SBR and the attached sidestream) during stage I were estimated and are shown in Figure 5.5c. At 100% synthetic feed, the observed biomass yield on VSS basis in the control-SBR and the modified-SBR systems were 0.41 and 0.145 mg VSS mg^{-1}COD^{-1}. Hence, the overall observed biomass yield in the modified system was almost 65% less

than the yield in the control system, when both SBRs were operated at 100 % synthetic feed. These numbers are consistent with our earlier findings (Huang et al., 2014; Datta et al., 2008) and results by other researchers (Novak et al., 2008; Chon et al., 2011). The observed biomass yield calculations for both systems account for sludge wastage during sampling and biomass present in the final effluent. The yield calculation for the control-SBR system also accounts for the conventional digester attached to it. As the feed composition to both SBRs steadily changed to the primary effluent, the overall percentage of sludge reduction, which was 65% with 100% synthetic feed, slowly decreased to 49%, during the transition and to 39% with 100% primary effluent. When changed to raw wastewater, the sludge production in the modified system was 35% less than in the control system (Figure 5.6c). These results demonstrate that the feed composition truly plays a crucial role in the sludge reduction mechanisms.

Phosphorus Mass Balance

Figure 5.7a and b present a mass balance of total phosphorus in the modified system, during stages III and IV. In Figure 5.7, a line represents the P accumulation in the modified system with a slope corresponding to the net phosphorus loading, which also includes the sampling loss. The average P content in the modified-SBR increased 46%, when the influent changed from primary effluent (CVWRF) to raw wastewater (SBWRD). The differences increased, when compared to the mass of P in the sidestream reactor. Comparing the expected P accumulation to the mass of P in the modified-SBR, sidestream reactor (attached to modified-SBR) and the accumulated wasted amount, there were 195mg and 340mg of P lost during stages III and IV respectively, representing 18% and 19% of the expected P accumulation.

Discussions

Reactor Performance in Terms of Nutrients Removal

In general, both SBRs performed well for COD, NH_4^+-N, and PO_4^{3-}-P removal. Based on the solids wasted from the modified-SBR, the solid retention was calculated to be close to 175 days. The control-SBR was operated at a standard SRT of 10-days. It is interesting to note that the modified-SBR performed exceptionally well for EBPR, despite the fact that the suggested optimum SRT for efficient EBPR is 5 to 15-days (Fukase et al., 1985; Shao et al., 1992; Rodrigo et al., 1996).

The overall PO_4^{3-}-P removal in all stages in both SBRs was always above 85%, except that the net P release at the end of the respective anaerobic periods steadily decreased, as the percentage of real primary effluent in the feed to both reactors increased. Perhaps, this is related to the amount of rbCOD present in the influent. The synthetic feed contained acetate to simulate the COD (365 mgL^{-1}) in the influent to both reactors. On the contrary, there was around 109 mgL^{-1} and 145 mgL^{-1} rbCOD present in the primary effluent and raw influent. As a result, as the influent to both SBRs changed to the real wastewater, possibly the amount of intracellular polymers accumulated inside PAOs also decreased, because the fraction of rbCOD present in the influent decreased. The concentration of polymer accumulation depends upon the amount of rbCOD (Vollertsen et al., 2006), and, consequently, the P released also decreased over time. Similar correlations between rbCOD and P-release have been reported previously (Martinez et al., 2001). Both systems achieved good phosphorus removal, as evidenced in the COD/P ratio of 100, which agrees with other reports (Randall et al., 1992; Lee et al., 1997), and the low COD/P ratio may positively affect the overall phosphorus removal

efficiency.

Both SBRs showed very efficient NH_4^+-N removal, during the entire experimental period. Occasional NO_2^--N accumulation was recorded in the control-SBR, especially during stages I and II (Figure 5.3e and b). Transient nitrite accumulation in the control-SBR demonstrates that the second step of biological nitrification was not complete. Several factors including temperature (Shammas, 1986; Antoniou et al., 1990), pH (Painter et al., 1983; Antoniou et al., 1990), and HRT (Li et al., 2013) can affect the nitrite oxidation by NOBs. When compared to the modified-SBR, none of these factors seems to be different for the control-SBR. The more efficient nitrification in the modified-SBR was perhaps due to the fact that the nitrifying population in the modified-SBR was more robust. However, incomplete nitrification also appeared during stage IV in both SBRs. The high practical COD from raw wastewater might be limiting ammonia oxidation rate, which is the rate-limiting step in nitrification, due to the lowering the oxygen diffusion of AOB (Zhong et al., 2015).

The difference in NO_3^--N concentration at the end of the aerobic cycle and at the end of the anoxic cycle indicates nitrate reduction, possibly through biological denitrification. As stated previously, COD was completely consumed during the anaerobic phase followed by the aerobic phase, leaving no appreciable carbon source for the denitrification to occur, during the last anoxic phase of each cycle. Hence, the possibility of biological nitrate reduction to reduced forms of nitrogen did not seem feasible. Denitrifying Polyphosphate-accumulating organisms (DNPAOs) (Saito et al., 2004) are a special class of PAOs that have recently caught attention of many researchers. DNPAOs use nitrite or nitrate instead of oxygen as an electron acceptor to remove

phosphorus without any extra-cellular carbon substrates under anoxic conditions (Saito et al., 2004; Meinhold et al., 1999). Another observation is that around 2.5 mgL^{-1} of NO_3^--N disappeared during the each anaerobic cycle. The possible explanation could be the occurrence of denitrification of nitrate to other reduced forms of nitrogen and/or and dissimilatory nitrate reduction to ammonium (DNRA) (Sgouridis et al., 2011).

Solids and Biomass Yield

The average TSS and VSS concentrations in the control-SBR were steady and remained nearly constant, irrespective of the feed composition. However, the TSS and VSS concentrations in the modified-SBR started increasing. The modified-SBR has an overall sludge yield of 0.11 mg VSS/mg sCOD, when it was operated with 100% synthetic wastewater. Hence, this SBR operated at an observed biomass wastage rate equivalent to 0.11 mg VSS/mg sCOD sludge yield. When the operation of this SBR was changed to real wastewater in a step-wise manner, the overall biomass production rate (measured as observed yield) increased, as shown in Figure 5.4c. However, the biomass wastage from the modified-SBR was still equivalent to 0.11 mg VSS/mg sCOD. As a result, the biomass in the modified-SBR steadily accumulated, as reflected by TSS and VSS concentrations in stages I and II. One-time sludge transfer to the connected sidestream tank from the modified-SBR at the end of stage II and the wastage of the biomass equivalent to the observed yield (i.e., 0.34) were the measures, which made the modified-SBR become stable during stage III. More consistent sludge wasted (70-days SRT) during stage IV, results in more stable solids concentration in the modified system.

According to the Figure 5.4b and Figure 5.5b, sludge in the modified-SBR had a lower volatile fraction (VSS/TSS ratio) than in the control-SBR, during the entire

operational period. Novak (2007) concluded that, because of the lack of wastage in the Cannibal™ system, coupled with the low yield for this system, the volatile fraction in the Cannibal™ system would be lower than in other systems. This lower volatile fraction indicates the loss of VSS in the modified-SBR system and might indicate that iron accumulated in the sludge (Novak at al., 2007). The modified-SBR was operated at an observed yield of 0.114 mg VSS mg^{-1} COD^{-1} at 100% synthetic feed and during stages I and II, and at 0.34 mg VSS mg^{-1} COD^{-1} in stage III. Observed yield became 0.148 mg VSS mg^{-1} COD^{-1} at 100% raw wastewater. Unlike in many past studies at lab scale (Novak et al., 2007; Datta et al., 2008; Chon et al., 2011; Coma et al., 2013), the sludge minimizing modified-SBR was operated at observed solid's yield and the operation of the modified-SBR was sustainable for nutrient removal and solids reduction. This research demonstrated again that operating a reactor in fasting and feasting mode (anaerobiosis) at small solids yield rather than at no solids wastage (infinite SRT) to achieve solids reduction is not only sustainable but can also be combined with efficient nutrient removal (Datta et al., 2008; Huang et al., 2014).

In stage I the overall observed solids yield in the modified-SBR system was almost 65% less than the yield in the control-SBR, which agrees with previous studies (Novak et al., 2006; Datta et al., 2008; Chon et al., 2011). When the percentage of real wastewater in the influent to both SBRs increased, the biomass yields increased in both SBRs, in which case, 0.541mg VSS mg^{-1} COD^{-1} biomass yield obtained in control system was close to the other studies with similar setups (Coma et al., 2013). VSS/TSS ratios in the control-SBR were 0.95 during stage I and decreased to 0.86 during the remaining stages. The percentage of sludge reduction in the modified-SBR decreased, as the

percentage of real wastewater in the feed increased from 25% to 100%. The VSS/TSS ratio in the modified-SBR decreased from 0.85 to 0.78, when the real primary effluent increased from 25% to 50% in the feed. The differences between TSS and VSS are inert TSS (iTSS) (Metcalf and Eddy, 2004). The low VSS/TSS ratio and high TSS value in the modified-SBR during stage II represent the accumulation of inert materials, perhaps present in the real wastewater. However, the VSS/TSS ratio in the modified-SBR was much lower than that in the control-SBR, which supports the loss of VSS in the modified-SBR (Novak et al., 2008). Nevertheless, the sludge minimizing modified-SBR was able to reduce the biomass and enabled 35-40% less sludge,s compared to the control-SBR with 100% wastewater.

Phosphorus Mass Balance

This study recovered over 80% of the total phosphorus, which was higher than the previous study. Goel and Noguera (2006) found 33% P loss by using persulfate digestion (APHA-AWWA-WPCF, 1985) in a similar system. The possible reasons for this unaccounted for P could be that the analytical methods used to measure total phosphorus were inadequate to recover all the phosphorus present in the sludge. However, the most likely reason is that the sidestream reactor encountered some conditions in which the chemical phosphorus precipitation (i.e., struvite) and the sampling did not truly represent the contents of the precipitation. The raw wastewater easily contained more biodegradable sludge than the primary effluent, resulting in more PO_4^{3-}-P being released, during stage IV in the sidestream reactor, than during stage III. This PO_4^{3-}-P released indicated that the hydrolysis of large organic molecules and degradation of amino acids and sugars occurred to produce the VFA. To date, the world's phosphorus sources are

being depleted at an alarming rate. We will run out of known phosphorus reserves in around 80 years, if the current consumption levels are maintained (Schröder et al., 2011). In future practice, this phosphorus-rich supernatant from the sidestream reactor can be combined with downstream ammonia-rich filtrate to form struvite by adding additional magnesium or calcium salts for the nutrient recovery. The supernatant with low nutrient can then be recycled into the mainstream process.

Summary

This chapter demonstrated the possibility of simultaneous sludge reduction and nutrient removal, using real wastewater. Following are the key observations:

1) Simultaneous biomass minimization and nutrient removal could be sustained in the laboratory reactor with synthetic as well as real wastewater.
2) Slightly higher P removal and more complete nitrification rates were recorded in the sludge minimizing modified-SBR, than those in the control-SBR.
3) With synthetic wastewater the modified-SBR generated 65% less biomass than in the control-SBR. The modified-SBR yielded 49% less biomass than in the control-SBR, during transition from the synthetic wastewater to the primary effluent. When both SBRs were at steady state with 100% real wastewater, the modified-SBR was able to achieve 39% and 35% sludge reduction with respect to the control-SBR with primary effluent and raw wastewater, respectively.
4) The measurement of total phosphorus could account for 80% of the total phosphorus loading, which might be due to the chemical precipitation in the modified system.

Future efforts should focus on ecophysiology of the key microbial community.

Also, denitrification due to the presence of DNPAOs and DNRA remains a topic of further investigation under similar settings.

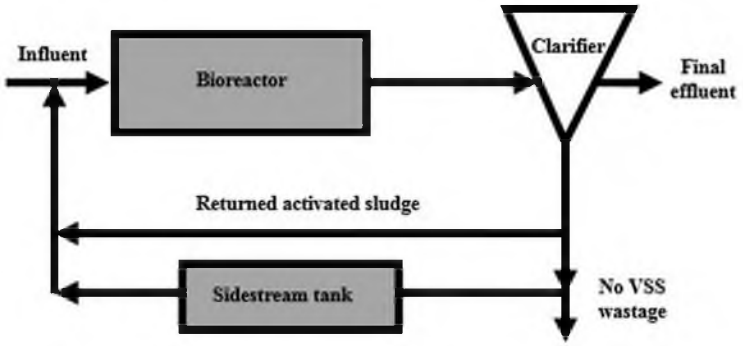

Figure 5.1: Schematic of a typical sludge minimizing activated sludge process through returned biomass fasting (in the sidestream tank) and feasting (in the bioreactor).

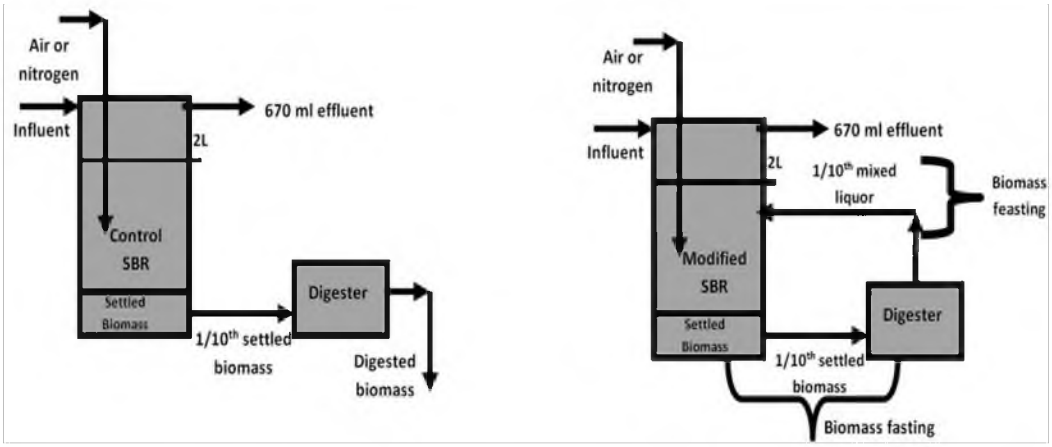

Figure 5.2: Schematics of the control (left side) and the modified (right side) SBRs

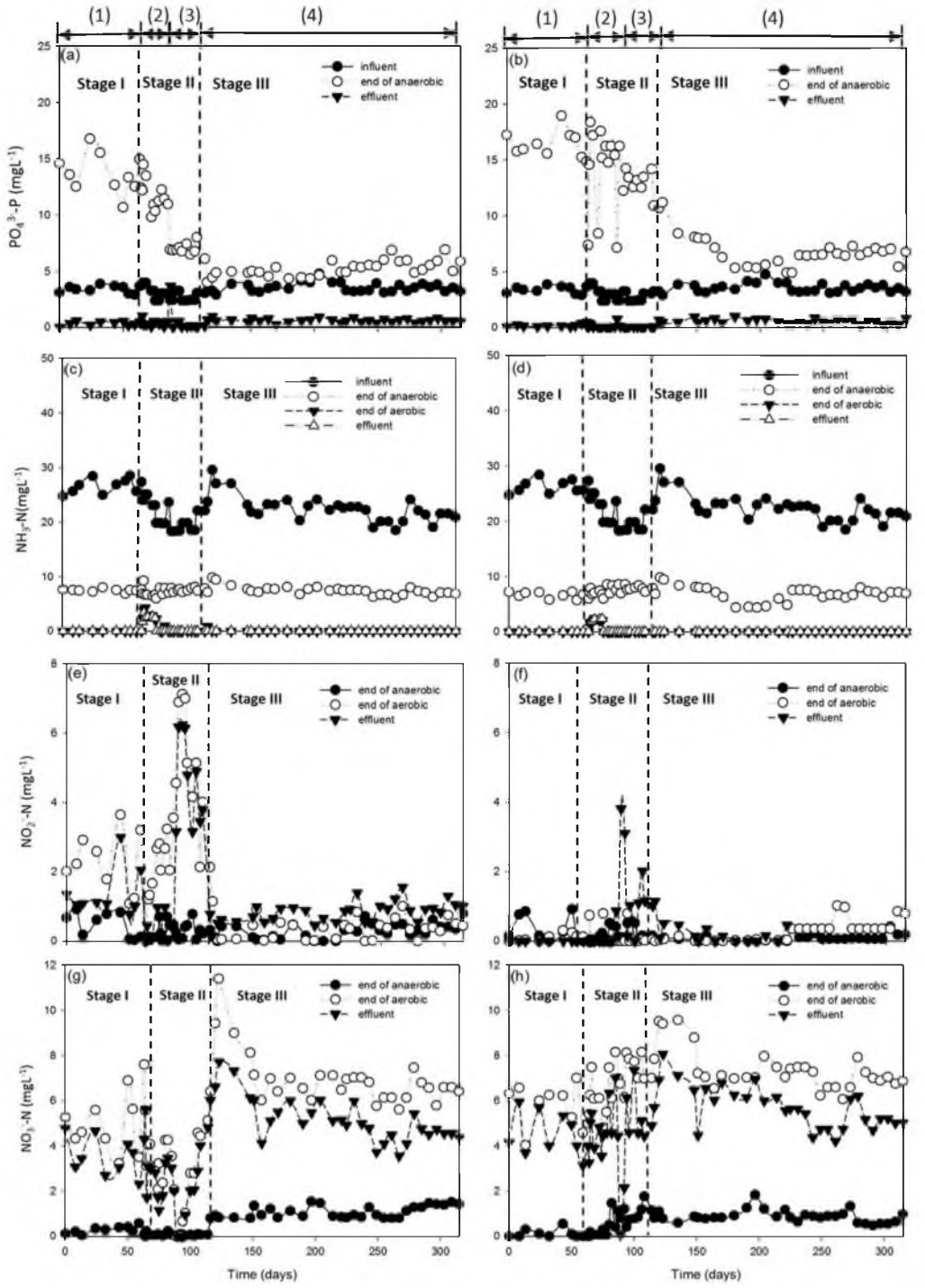

Figure 5.3: Changes in PO_4^{3-}-P, NH_4^+-N, NO_2^--N and NO_3^--N mg/L in the control-SBR (a, c, e, g) and the modified-SBR (b, d, f, h), respectively. Stage I, II, and III refer different influent conditions. More specifically, (1) (i.e., Stage I) with 100% synthetic wastewater, (2) and (3) (i.e., Stage II) with 25/75 (v/v) and 50/50 (v/v) of synthetic wastewater/wastewater, respectively, and (4) (i.e., stage III) with 100% wastewater.

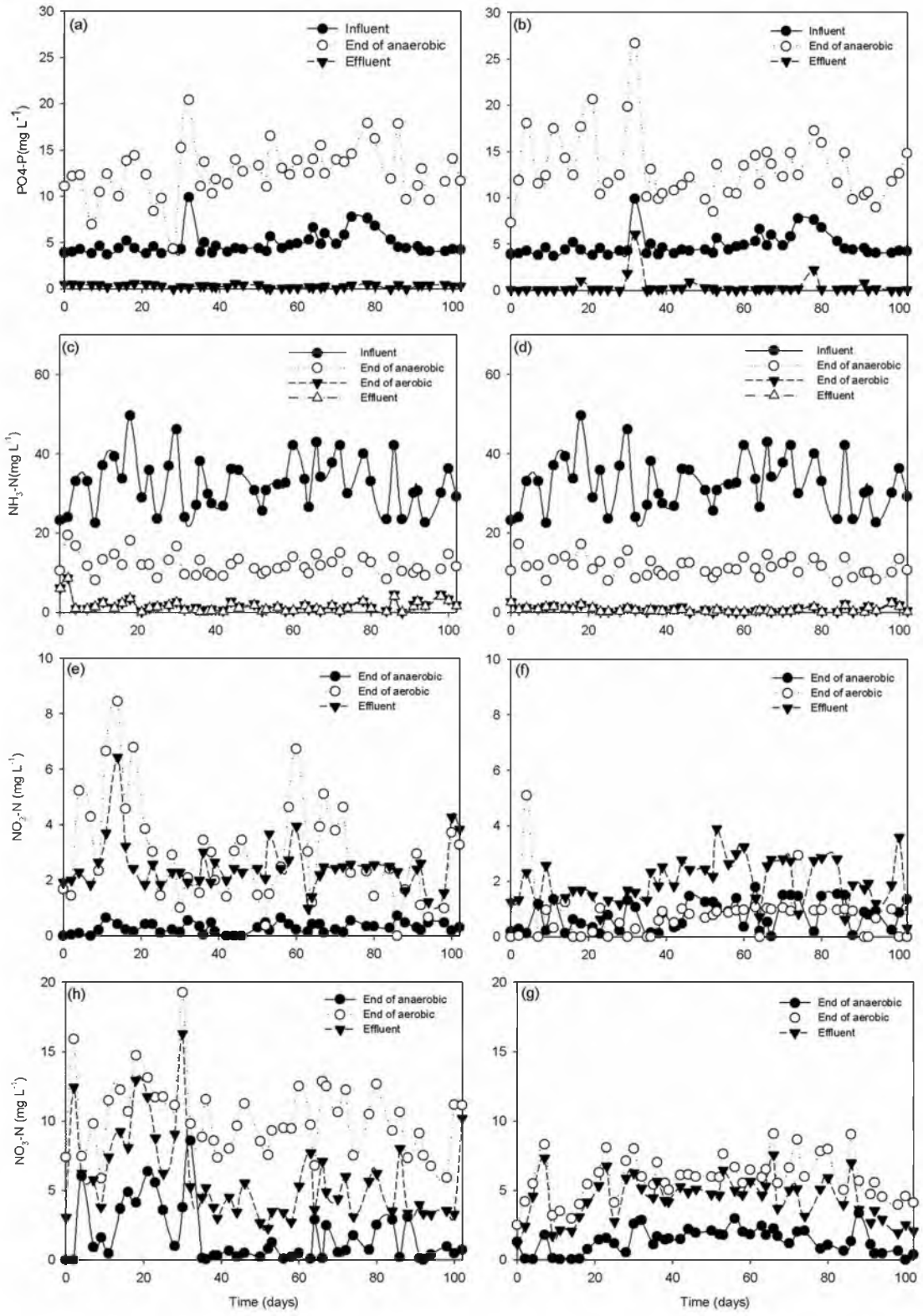

Figure 5.4: Changes in PO_4^{3-}-P, NH_4^+-N, NO_2^--N and NO_3^--N mg/L in the control-SBR (a, c, e, g) and the modified-SBR (b, d, f, h) in stage IV, respectively.

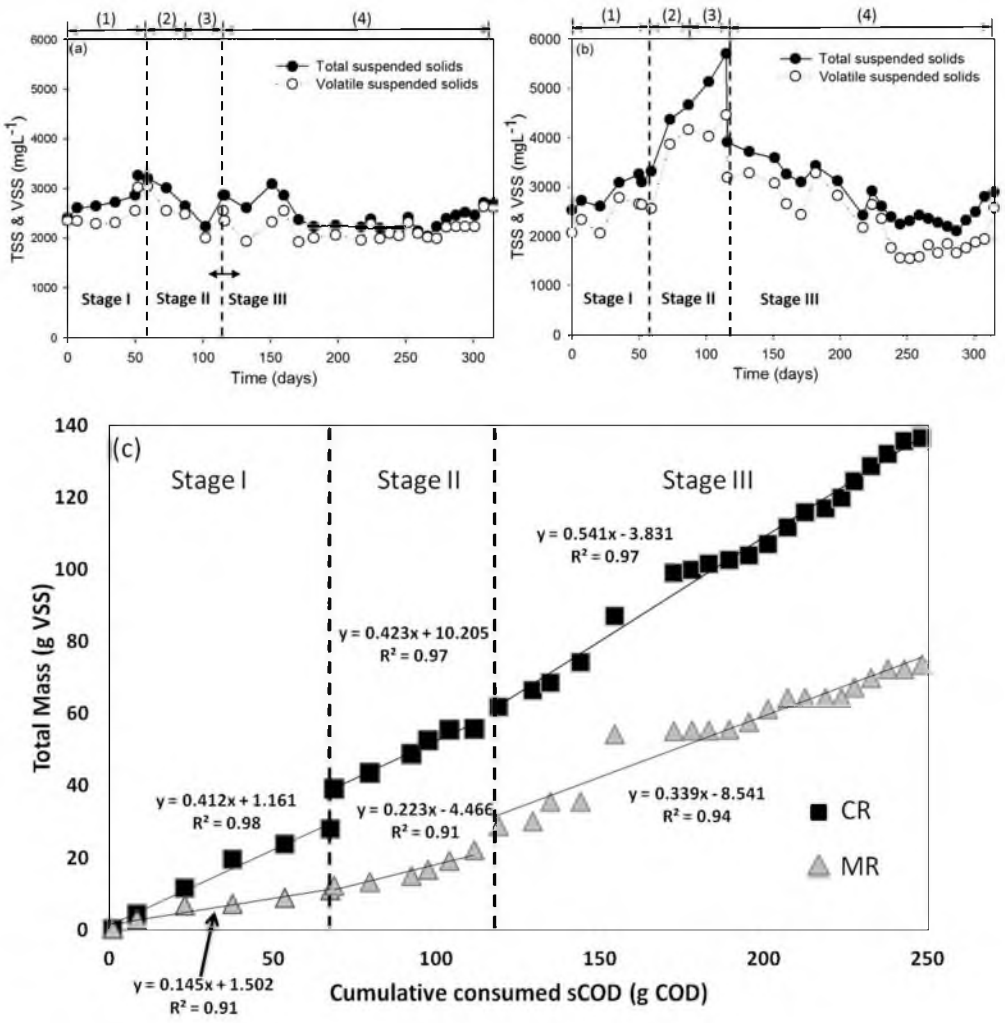

Figure 5.5: Total and volatile solids in (a) control-SBR and (b) modified-SBR. (c) The observed sludge yields from (CR): control-SBR with CHT and (MR): modified-SBR with MHT during the whole experiment period changed to primary effluent. Stage I, II and III refer different influent conditions. More specifically, (1) (i.e., Stage I) with 100% synthetic wastewater, (2) and (3) (i.e., Stage II) with 25/75 (v/v) and 50/50 (v/v).

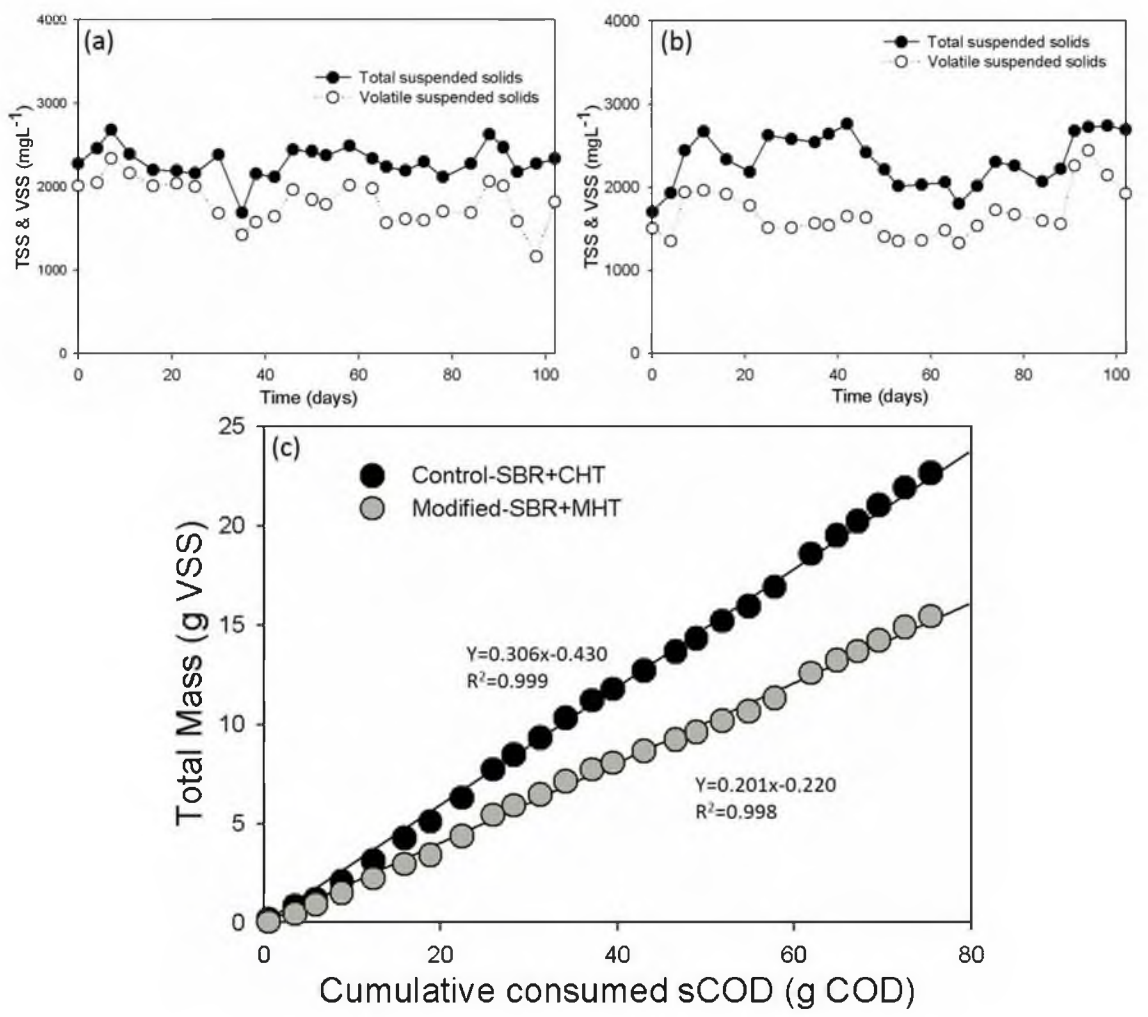

Figure 5.6: Total and volatile solids in (a) control-SBR and (b) modified-SBR. (c) The observed sludge yields from (CR): control-SBR with CHT and (MR): modified-SBR with MHT during stage IV.

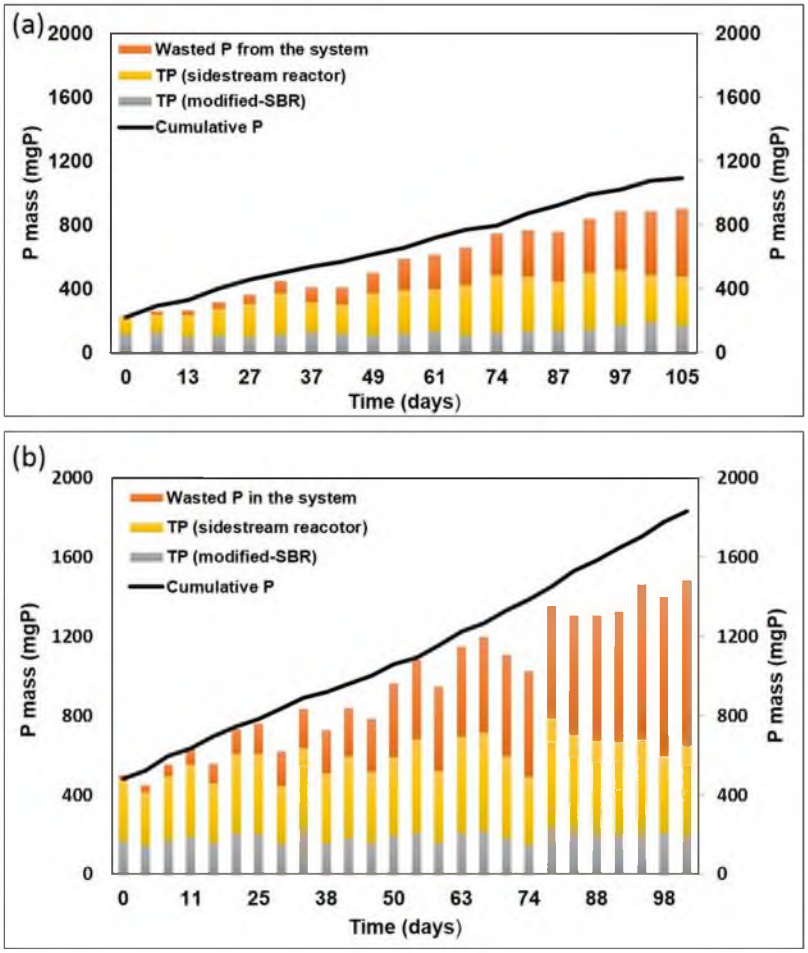

Figure 5.7: The phosphorus mass balance in modified system during stage III (a) and IV (b).

THE MICROBIAL COMMUNITIES ANALYSIS OF ACTIVATED SLUDGE FROM A LAB-SCALE SIMULTANEOUS NUTRIENTS REMOVAL AND SLUDGE MINIMIZATION REACTOR[***]

Abstract

The biomass fasting and feasting process has been evolved in coupling sludge minimization with nutrients removal process in recent years. In this study, the next generation sequencing (Illumina Miseq) was performed to compare microbial communities between two lab scale sequencing batch reactors. First, in one sludge minimization (called modified-SBR) and the other in conventional activated sludge (referred to as control-SBR). Modes were started and operated with synthetic feed then changed to real primary effluent from different local full scale wastewater treatment plants.

Illumina Miseq analysis revealed *Proteobacteria* and *Bacteroidetes* were two predominant phyla in all samples. Canonical correspondence analysis (CCA) results indicated that the bacterial community variance correlated most strongly with concentration of readily degradable chemical oxygen demand (rbCOD), NH_4^+-N, total

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COD (TCOD) and the solids retention time (SRT). Possible mechanism of sludge reduction of modified-SBR contains more slow growing bacteria (*Nitrospira, Mesorhizobium, and Candidatus Accumulibacter*) and filamentous bacteria (unclassified *Cytophagales*). When 100% of real primary effluent became influent, TCOD was the major factor that shaped the microbial community. One the other hand, both SBRs showed a greater diversity of ammonia oxidizing bacteria (AOBs) with real wastewater. The nitrite oxidizing bacterial community and the polyphosphate accumulating organisms (PAOs) responded similarly in both SBRs. Two *Dechloromonas*-related OTUs were detected in both SBRs as the denitrifying PAOs to utilize nitrite or nitrate to remove phosphorus without any extracellar carbon substrates under anoxic conditions.

Introduction

Activated sludge process (ASP) has been widely used for both municipal and industrial wastewater around the world due to its efficient removal of organic matter, nitrogen and phosphorus. Although it is a highly efficient process for the removal of nutrients, one of its drawbacks is high sludge production (Saby et al., 2003). Compared to other sludge reduction at source strategies, the Cannibal™ process (also known as the biomass fasting and feasting process) had more positive effects (Guo et al., 2013; Böhler and Siegrist, 2006) because 1) it did not require no extra chemical or physical addition; 2) it can improve of the sedimentation ability; 3) it can capable with treating complex components or high strength organic pollutants; 4) it is flexible to operate and easy to be meliorated as well as 5) it is economic efficiency and environmental friendliness. Previous studies demonstrated that lab-scale processes similar to the Cannibal™ could reduce 20-65% of the sludge (Coma et al., 2013; Novak et al., 2006; Chen et al., 2003;

Saby et al., 2003). Additional, it could also occur with nutrients removal (Huang et al., 2014; Datta et al., 2009; Goel and Noguera 2006).

Although these earlier studies showed feasibility of lab-scale processes similar to the Cannibal™ in ASP, the microbial communities in these processes was not clear. The information on microbial communities in the Cannibal™ process is useful and can help engineers and practitioners to optimize the process with nutrients removal and also provide a deeper understanding of this sludge reduction mechanism. Our studies assessed the microbial composition and its relationship to nutrients removal performance in the Cannibal™ process and similar ones (Huang et al., 2014). Previous studies mainly focused on particular bacteria, such as ammonia oxidizing bacteria (AOB), nitrite oxidizing bacteria (NOB), or polyphosphate accumulating organisms (PAO), but lacked an overview of the microbial communities' features of this process. Only Kim et al. (2012) showed the bacterial communities in a similar process which was analyzed by polymerase chain reaction-denaturing gradient gel electrophorese (PCR-DGGE). The results indicated that a sidestream reactor was primarily related to conventional anaerobic digesters, but there was a large number of DGGE bands in the system that were affiliated to certain bacteria that could not clearly explained.

Higher than 99% of the microorganisms in the world cannot be cultivated by conventional culturing methods (Liaw et al., 2010). Most of the time, traditional approach involves amplification of target gene fragments using PCR, followed by building a library for sequencing. However, the problems of this approach include: 1) there are no "universal primers" for all taxa (include bacteria, archaea, fungi, and virus) and therefore optimal PCR could only obtain part of the biodiversity information; and 2) PCR

amplication efficiency would have been biased toward a limited number of taxa. Previous studies lacked tools with adequate coverage for profiling the whole complex microbial communities in the sludge minimization process through fasting and feasting. These results could be limited by the efficiencies of primers, taxonomic classification effectiveness of variable regions selected, and pyrosequencing noises (Fayle et al., 2013; Ju et al., 2014). They do not always fully reflect microbial diversity (Yu and Zhang, 2012).

To date, high-throughput sequencing methods, such as 454 pyrosequencing, Illumina sequencing, and Ion Torrent Personal Genome Machine (PGM) technologies have been recently applied on microbial communities of activated sludge from municipal wastewater treatment plant and some metagenomic studies on activated sludge have been reported (Sanapareddy et al., 2009; Yu and Zhang, 2012; Ju et al., 2014; Sheik et al., 2014). The next generation sequencing has completely changed our capability to sequence DNA and RNA in unlimited amounts. This approach provides a rapid and relatively precise identification and quantification of the composition of entire microbial communities in activated sludge. This technique can provide much greater scale and detail of sampled communities compare with conventional methods.

In this research, two lab scale sequencing batch reactors; one in sludge minimization (called modified-SBR) and the other in conventional activated sludge (referred as control-SBR) modes were started and operated with synthetic feed and then changed to 100 % real primary effluent from a local full scale wastewater treatment plant. The objectives of this study are to use high-throughput sequencing metagenomic technology (Illumina Miseq sequencing) as well as 16S rRNA gene traditional amplicons

techniques to compare microbial communities in these two reactors; these revealed (1) the changes of bacterial diversity in the biomass based on the nutrients removal and sludge reduction because of influent variation and (2) the existing mechanisms of sludge reduction. For the traditional molecular tools, we used *ppk1* genes as genetic biomarkers to investigate the ecology of PAOs; terminal restricted fragment length polymorphism (TRFLP) was used to investigate the identity of AOBs and NOBs.

Materials and Methods

Reactor operation and analytical analysis as the methodology for this study were explained earlier in the Chapter 5. Genomic DNA was extracted from biomass samples collected from the reactors in stage I, II and III. Additionally, DNA extraction was discussed in detail previously.

PCR and TRFLP for AOBs and NOBs

TRFLP for AOBs was performed using the modified protocol developed by Park and Noguera (2004). Briefly, primers were labeled forward (*amo*A-1F) and reverse (*amo*A-2R) primers and used to amplify the *amo*A gene (Park and Noguera, 2004). The forward primer was labeled with the fluorophore HEX, and the reverse primer was labeled with 6FAM. Amplification was done on a master gradient theromocycler (Eppendorf, NY) with the following temperature cycle: denaturation at 94°C for 5 min, followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 56°C for 1.5 min, and elongation at 72°C for 1.5 min, with polishing steps at 60°C for 1.5 min and 72°C for 10 min. PCR products were run on 1% agarose gel for 40 min against a standard DNA Ladder (Promega, WI) to verify the length. The products were then purified from the gel

using the Qiaex II gel extraction kit (Qiagen, Valencia, CA). The purified PCR products were digested with TaqI restriction endonuclease (MBI Fermentas, Hanover, MD). Restricted enzyme digested fragments were processed on an Applied Biosystems 3730 Genetic Analyzer capillary electrophoresis instrument (Applied Biosystems, Foster City, CA) at the University of Utah Core Facility (Salt Lake City, UT) and analyzed using the GeneMapper software, version 2.6 (Applied Biosystems, Foster City, CA). The resulting fragment lengths were compared with known fragment lengths of AOBs to identify presence of specific AOBs (Park and Noguera 2004; Park et al., 2002; Horz et al., 2000).

In case of NOBs, TRFLP was performed using the modified protocol developed by Siripong and Rittmann, 2007. The DNA was amplified using universal primers 8f (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492r (5'-GGYTACCTTGTTACGACTT-3') (Lane et al., 1991), and amplified again with a nested PCR using fluorescence-labeled specific primers targeting the 16S rRNA genes of NOBs-Eub338f 5'-ACTCCTACGGGAGGCAGC-FAM, Nit3r (5'-CCTGTGCTCCATGCTCCG-3') (*Nitrobacter* specific) and Ntspa 685Mr (5'- CGGGAATTCCGCGCTCCG -3') (*Nitrospira* specific), (Maixner et al., 2006). The final PCR amplification products were purified and digested with MspI (HpaII) restriction endonuclease (Promega, WI) at 37 °C for 3 h. Data processing and analysis of the terminal fragments were performed as described earlier for AOBs.

Quantification Ppk-Based Phylogenetic Clades

The qPCR to quantify *ppk1* gene copy numbers was conducted on a Realplex Mastercycler (Eppendorf, NY) using iQ^{TM} SYBR green supermix (Bio-Rad, Hercules, CA) with a total reaction volume of 20µL. A four-point calibration curve for qPCR was

produced by 10-fold serial dilution of in-house clone as positive control in triplicate within each assay, at 10^3 to 10^8 target copies per reaction. Primer sets were used to selectively target the *ppk1* gene representing each "*Candidatus* Accumulibacter" clades (He et al., 2007). All primers and the qPCR program for already established *ppk1* clades and qPCR conditions were obtained from He et al. (2007). Measurements of clade diversity and evenness within the "*Candidatus* Accumulibacter" lineage were calculated for individual samples by using the Shannon index ($H = -\sum P_i lnP_i$) (Shannon and Weaver, 1949) and Pielou regularity index ($R = H/lnS$), respectively (He et al., 2007).

Illumina Miseq Sequencing

About 400ng of each of the DNA samples were sent to the Research and Testing Lab (Lubbock, TX) to perform 16S rRNA gene PCR amplification, Illumina MiSeq sequencing, and data analysis. Primers 515F (5′ -GTGCCAGCMGCCGCGGTAA-3′) and 806R (5′ -GGACTACHVGGGTWTCTAAT-3′), which target V4 hyper variable regions of bacterial 16S rRNA genes, were selected to be used to assess bacterial community composition. These primers were universal for a broad range of bacteria and archaea, which could yield accurate phylogenetic information (Bates et al., 2011). The operational taxonomic unites (OTUs) that were defined as PAO, DNPAO, AOB, and NOB were separated from the final taxonomic information obtained from the Research and Testing Lab. These separated OTUs were used to generate a phylogenetic tree which was constructed by using MEGA version 6 software (Tamura et al., 2007). Cannonical correspondence analysis (CCA) was used to examine the relationships of bacterial communities and environmental variables. Based on partial redundancy analysis (RDA), variance-partitioning analysis (VPA) was performed to attribute the variation observed in

the bacterial communities to the environmental variables. CCA, RDA, and VPA were performed by the vegan package in R 2.14.0 (R Development Core Team, 2011). In this study, the pairwise statistical comparisons of the taxonomy in genus level between all of the samples in the same reactor were also carried out using STAMP (Park and Beiko, 2010).

Results

The Overview of Sequencing and Microbial Diversity

As shown in Table 6.1, after removing low quality sequences and chimeras, 34699-72392 effective sequences for 8 samples were extracted from both SBRs. A total of 1389 OTUs were recovered from these samples. To assess the internal complexity of individual microbial populations, the Shannon index (diversity) and the Pielou regularity index (evenness) were calculated. The diversity index provides more information about the richness of species, as well as the relative abundance of different species. Species evenness refers to how close in numbers each species is in an environment. The microbial communities in both SBRs became less diverse when 25% of real primary effluent was introduced. Then the value of these two indies started increasing when the percentage of real wastewater in influent increased (as Table 6.1 shows). Modified-SBR at stage III (i.e., 100% primary effluent) contained the most diversity and evenness in microbial communities compared to the others. Both diversity and evenness indies from this study were similar to other lab-scale bioreactors, but lower than that of the full-scale municipal wastewater treatment systems (Chu et al., 2015; Wen et al., 2015; Ma et al., 2015; Ibarbalz et al., 2013).

Bacteria Communities Analysis

In this study, all the sequences (over 99.95%) were assigned to bacteria and only a few sequences belonged to archaea. There were 24 phyla and nearly 62 classes identified in both SBRs, which was much higher than previous studies based on PCR-DGGE (Kim et al., 2012). Figure 6.1 summarized the relative bacterial communities abundance at the phylum level for each sample. The sequences that did not have any alignment hits against taxonomic bacteria database were categorized as ''unknown." Sequences that were unclassified at a particular taxonomy were labeled as 'unclassified'. *Proteobacteria* and *Bacteroidetes* (constituted over 77%) were the predominant phyla in each sample. Previously, it was also shown that *Proteobacteria* and *Bacteroidetes* were the prominent phyla in different lab-scale reactors and municipal wastewater treatment plants (Sánchez et al., 2013). These findings were comparable to this study. In control-SBR, the percentage of *Proteobacteria* decreased when the percentage of real wastewater as influent increased (stage I and II), then grew in stage III. The rises and drops of *Bacteroidetes* in control-SBR corresponded to drops and rises of *Proteobacteria*.

Bacteroidetes reached the highest percentage while *Proteobacteria* reached the lowest percentage when the influent was 50/50 (v/v) synthetic/real wastewater. However in stage III, the portion of *Bacteroidetes* was higher and *Proteobacteria* of control-SBR than the percentages founded in stage I. On the other hand, the percentage of these two major phyla stayed similar in modified-SBR throughout the experiment period. Additionally, these percentages of the two major phyla were closed to those in control-SBR in stage III. Subdominant phyla include *Unknown Bacteria* (0.62-10.13%), *Acidobacteria* (0.24-8.63%), *Chloroflexi* (0.05-2.02%) and *Nitrospirae* (0.01-1.58%).

Interestingly, the modified-SBR always contained a higher percentage of *Unknown Bacteria* and *Nitrospirae* than in the control-SBR.

Figure 6.2a and b summarized the major classes under the *Proteobacteia* and *Bacteroidetes*, respectively. *Betaproteobacteria* was the largest class (25.70-79.73%) in most of the samples, and it decreased in both SBRs when the amount of real wastewater as influent increased (Figure 6.2). *Betaproteobacteria* were found to be highly versatile in pollutant degradation capacities and detected in various activated sludge process systems such as domestic wastewater, phenol-containing wastewater, and coking wastewater treatment systems (Figuerola and Erijman, 2007; Wang et al., 2012). When the feed was switched to 50%, control-SBR contained the smallest portion of *Betaproteobacteria*, and the reduction of *Betaproteobacteria* corresponded to the rise of *Sphingobacteriia* (phylum *Bacteroidetes*) (As shown in Figure 6.2). Followed by the *Betaproteobacteria*, *Alphaproteobacteria*, *Gammaproteobacteria* and unclassified *Proteobacteria* were less predominant classes within *Proteobacteria* phylum in all of the samples (Figure 6.2a). In both SBRs, the percentage of *Alphaproteobacteria* stayed similar, while *Gammaproteobacteria* increased when the percentage of real primary effluent in influent was increased. Within phylum *Bacteroidetes* (Figure 6.2b), there were *Cytophagia*, *Sphingobacteriia, Flavobacteriia,* and *unclassified Bacteroidetes* as major classes. However, it differed when control-SBR fed with 50% primary effluent contained approximately 30% class *Sphingobacteriia*, other samples did not have higher than 10% of any classes under *Bacteroidetes*.

Relationship of environmental factors to the bacterial communities. The major genera in all samples were summarized in Table 6.2. Within *Proteobacteria, Acidovorax,*

Dechloromonas, Rhodocyclus, Thauera, Zoogloea, and *Candidatus Accumulibacter* were primary shared genera in all of the samples (Table 6.3). *Unclassified Sphingobacteriia* and unclassified *Cytophagia* were the predominant genera under the phylum *Bacteroidetes*. To further investigate the changes of these major genera correlated to the operational and environmental factors, CCA was performed. Six environmental variables were selected: influent concentration of rbCOD, TCOD, NH_4^+-N, PO4-P, and TSS, as well as SRT. In CCA ordination diagram (Figure 6.3), the length of an environmental parameter arrow in the ordination plot indicates the strength of the relationship of that parameter to community composition.

From a close examination of Figure 6.3 several observations could be made. First, we can appreciate a very prominent separation between samples in different operational mode and fed with different influent. All of the samples were separated into four clusters (Figure 6.3):

- Cluster I: This cluster included two samples from control-SBR (synthetic wastewater and 25% real primary effluent).
- Cluster II: It contains two samples from modified-SBR (synthetic wastewater and 25% real primary effluent).
- Cluster III: Control-SBR fed with 50% primary effluent was found in this cluster.
- Cluster IV: the remainders of the samples were incorporated in this fourth cluster.

Second, rbCOD, TCOD, NH_4^+-N and SRT seem to be the strongest influences on the bacterial communities' composition in the SBRs from all of the parameters. NH_4^+-N and rbCOD had a strong positive relationship with the bacterial communities from cluster I and four genera (marked as dark yellow in Figure 6.3 and Table 6.2). The percentage of

these four genera decreased in all samples when the rbCOD/ammonia concentration in influent decreased. SRT had a significant effect on the bacterial community in cluster II as well as night genera (marked as red in Figure 6.3 and Table 6.2). Within these night genera, except *Nitrospira*, the percentage of other genera decreased when SRT decreased. Cluster III, IV, and 19 genera (marked as black in Figure 6.3 and Table 6.2) were distributed either along or close to the TCOD. The portion of most of these genera increased when the TCOD increased during time the real primary effluent was introduced.

VPA was further performed to assess the contributions of influent characteristics (COD, N, TP, solids concentration) and operational parameters (SRT, MLSS) to the whole microbial community variance. This test indicated that operation parameter and influent characteristics could independently explain 30.1% and 63.6% of the variation of bacterial communities, respectively.

AOBs and NOBs Communities

Ammonia and nitrite oxidizing community using TRFLP. TRFLP targeting the *amoA* gene for AOBs (Figure 6.4a and b) and 16S rRNA gene based TRFLP for NOBs (Figure 6.4c and d) were conducted for both SBRs at different time periods. Panels in Figure 6.4 present TRFLP modified-SBRs. In the TRFLP PCR reaction, both forward and reverse primers were labeled. Therefore, each TRFLP electropherogram shows a forward (blue) and a reverse (green) terminal fragment for each AOB. When the SBRs were operated with the synthetic wastewater, several peaks were detected as shown in Figure 6.4a and b. The control-SBR was found to be dominated by AOBs belonging to *N. oligotropha* (terminal fragments: 48/135) and *N. europaea/eutropha* lineages (terminal

fragments: 219/270) (Horzet al., 2000; Park and Noguera, 2008). For the modified SBR, there were 48/135, 48/354, and 48/441TF peaks indicating the presence of *N. oligotropha*, *N. cyrotolerans* and *N. marina* related AOBs.

When the feed to both reactors was switched to 25 % real primary effluent, the TF of size 219/270 representing *N. europaea/eutropha* became more prominent and the trend continued when the reactors were fed with higher percentages of real primary effluent. This lead to 100 % primary effluent in stage III especially for the control-SBR. However, the TF (48/135) belonging to *N. oligotropha* disappeared right after the feed was changed to the control-SBR. In the modified-SBR, TRFLP showed a much more diverse community of AOBs during the transition period. Terminal fragments (48/441) representing *N. cyrotolerans* and *N. marina*-related AOBs were observed in the modified-SBR. Furthermore, TF combination of 491/491 was also seen in the modified-SBR (Figure 6.4b). This terminal fragment could represent several groups of AOBs, including *N. communis, N. oligotropha, N. europaea/eutropha*, *N. cyrotolerans* and *N. marina* (Sirpong and Rittmann, 2007). However, the 491/491 TF peak slowly disappeared in the modified-SBR as the feed composition was slowly changed to 100 % real primary effluent. Finally, like in the control-SBR, AOBs seemingly related to *N. europaea/eutropha* were active in the modified-SBR as well. These result agrees with the results generated by Datta et al. (2010), where these researchers studied the seasonal variations in the AOB community in Central Valley Water Reclamation Facility (CVWRF, Salt Lake City, UT) mixed liquor and found that *N. europaea/eutropha*-related AOBs dominated regardless of season in the CVWRF. It is worth mentioning that the primary effluent to feed the lab scale reactors was obtained from CVWRF.

Panels c and d in Figure 6.4 shows TRFLP profiles for nitrite oxidizers (*Nitrospira*) in the control and the modified SBRs respectively. It is evident from these panels that *Nitrospira* (TF=277 bp) related NOBs were prevalent in both SBRs. However, when the feed was slowly changed to the real wastewater, the TF of size 333 also started showing up, and by the time the reactors were operating with 100 % real wastewater, the 333 bp TF had a much stronger signal. This specific TF (333 bp) belongs to one of the *Nitrospira moscoviensis* strains. TRFLP was also performed using *Nitrobacter*-related NOBs. TRFLPs profiles enabled weak signals indicating that either *Nitrobacter* realted NOBs were not present or were not the key players with both types of wastewaters.

Ammonia and nitrite oxidizing community using Illumina Miseq. Overall, the percentages of both AOB and NOB were below 5% in all the samples from the result of Illumine Miseq sequencing. Compared with the percentages of AOB and NOB in stage I from both SBRs, they were all significantly increased in stage III. In order to further identify the AOB and NOB, nine OTUs were isolated to compare the phylogenetic differences from these two reactors during the influent changed and other relevant sequences from publicly available databases as depicted in Figure 6.5 and Figure 6.6. AOB has been postulated as the main contributor to ammonia oxidation, of which *Nitrosomonas* and *Nitrosospira* are the most important genera, were investigated in the ASP (Wells et al., 2009). In this study, only one genus of AOB, *Nitrosomonas,* was detected in all of the samples represented by six OTUs. The OTUs as follows:

- OTU 829 was 100% identified as *N. stercoris* which was found in the high ammonia compost (Nakagawa and Takahashi, 2015).
- OTU1226 was 99% identified as *N. europea* sequence (Chain et al., 2003).
- OTU273 was 100% identified as a clone which was found in a partial

nitrification/anammox process from the aerobic tank (Prachakittikul et al., 2014).

- OTU511 was 100% identified as *N. ureae* strain Nm10 (Yarza et al., 2015).
- OTU1254 was 100% identified as Nitrosomonas sp. which was isolated from an ammonia-oxidation isolated Nm86 strain (Purkhold et al., 2003).
- OTU875 was 100% identified as *N. oligatropha* which was from an enrichment culture in activated sludge couple with Mn(II) oxidation and nitrification (Cao et al., 2015).

When the feed to both SBRs were synthetic wastewater and 25% primary effluent, the AOBs found in control-SBR were belonged to *N. oligotropha* (OUT 875) and *N. europaea* (OTU1226), while in modified-SBR were identified as only *N. oligotropha* (OUT 875). These results were similar to the TRFLP results mentioned above. However, as the influent changed to 50% and 100% primary effluent, the AOBs became more diverse in both reactors. Finally, there was no *N. oligotropha* related OTU found in both SBRs, and modified-SBR contained more diverse AOBs than in control-SBR in stage III (Figure 6.5).

For NOB, only two genera (*Nitrospira* and *Nitrobacter*) were detected, represented by three OTUs (as shown in Figure 6.6): OTU525 (100% identity to *Nitrospira* species from activated sludge in Japan (Fujitani et al., 2014)), OTU614 (100% identity to *Nitrospira* species from opalinus clay borehole water in Switzerland (Bagnoud et al., 2015)), and OTU533 (100% identity to *Nitrobacter* species from a burned native tallgrass priarie in Kansa (Jangid et al., 2010)). Among these three OTUs, OTU525 (*Nitrospira* sp.) was more predominant in all of the samples. The percentage of Nitrobacter sp. (OTU533) was less than 0.1%, which matched the TRFLP results. This indicated the *Nitrobacter*-related NOBs might not be the key factor in both SBRs in the nitrite oxidizing step. Another observation from Figure 6.6 is when the portion of primary

effluent as influent increased the percentage of OTU525 (*Nitrospira* sp.) also increased in all of the samples. Table 6.2 also shows that the percentage of *Nitrospira* was found to be more in modified-SBR than that in control-SBR during the whole operational period.

PAO and DNPAO Communities

PAO and DPAO community using Illumina Miseq. An important task was to identify the PAO responsible for phosphorus removal in both SBRs. For PAO, the genus *Candidatus* Accumulibacter was identified in Table 6.2. The abundances of *Candidatus* Accumulibacter genus were 4.81-12.28% and 10.65-52.14% in the control-SBR and modified-SBR, respectively (as shown in Table 6.2). The average reported PAO percentages were 6-22% (Lv et al., 2014); this ratio in control-SBR belonged to this range but modified-SBR with 0-25% of wastewater was apparently much higher than that. As previously described, *Candidatus* Accumulibacter was the most frequently reported and solely well-accepted PAO in the activated sludge system. There was only one OTU (OTU1380) in the samples assigned to *Candidatus* Accumulibacter-related sequences. According to nitrate and nitrite reduction during the anoxic period without appreciable carbon source as described previously, it was possible that DNPAO may exist in both SBRs. To identify DNPAOs, OTU1256, and OTU1319 came to the fore-front because of its high similarity with previous reported DNPAOs from lab-scale SBR and full-scale systems (Lv et al., 2014).

The phylogenetic relationship between OTU1380, OTU1256, OTU1319, *Dechloromonas*-related PAO, the *Candidatus* Accumulibacter-related PAO and selected glycogen accumulated organisms (GAO) was shown in Figure 6.7. OTU1380 was 100% similar to a sequence was found in a SBR with N and P removal (Kim et al., 2013). The

percentage of OTU1380 in the control-SBR was found to be the lowest when the 25% real primary effluent was introduced, then the number recovered and increased when the real primary effluent in influent increased. All selected environmental sequences related to OTU1256 fell in a cluster that was related more to an uncultured *Betaproteobacterium*, which was found in a nitrogen and phosphorus rich lake water sample from China (Li et al., 2012). OTU1319 was 98% identified as an uncultured *Dechloromonas* sp. which was found in a membrane bioreactor (Fang, 2011). OTU1256 and OTU1319 also shared similar sequences in the V4 region to *Dechloromonas*-related organisms from a full-scale EBPR clone (DQ640664) identified by Kong et al (2007). Control-SBR with synthetic wastewater and 25% of primary effluent as influent contained the higher percentage of OTU1319 and OTU1256 than these in the modified-SBR, but then these percentage became similar in both SBRs (Figure 6.7). The presence of GAO is known to potentially compete with PAO due to its uptake of volatile fatty acid (VFA) under anaerobic conditions, but not the accumulation of polyphosphate under aerobic conditions (Kim et al., 2011; Kondo et al., 2007). There was no GAO found in all of the samples against with Basic Local Alignment Search Tool (BLAST) with a local database containing 48 known GAO sequences (Lv et al., 2014).

Quantification of CAP-related PAO. The *ppk1* gene is the genetic biomarker to detect all currently defined *Candidatus* Accumulibacter clades (He et al., 2007). Table 6.3 summarizes different *ppkI* clades quantified during the experimental run under different feeding scenarios. The total "*Candidatus* Accumulibacter" *ppk1* abundance was calculated as the sum of the *ppk1* abundances detected from the five "*Candidatus* Accumulibacter" clades (He et al., 2007). The clade IIB was not detected in both SBRs

across the whole experimental period. As *ppk1* is a single-copy gene in "*Candidatus* Accumulibacter" (He et al., 2007), its abundance can represent the cell abundance of this organism. The *ppk1* based phylogenies results clearly show the dominance of clade IIC in both SBRs (Table 6.3) under all feeding conditions. In stage I, clade IIC constituted around 90% of total *ppkI* gene abundance in both SBRs. Except during the period when the SBRs were fed with 50 % real and the rest with synthetic feed, clades IIC and IID dominated in both SBRs. When the SBRs were fed with 50% real wastewater, clade IIC increased to almost 93% and clade IID decreased to 1.5% in the modified-SBR. However, these percentages again returned to around 82% and 11.2% when the modified SBR was fed with 100% real primary effluent.

To compare the "*Candidatus* Accmulibacter" clade diversity and evenness in both SBRs and different influent characteristics, the Shannon index (diversity) (Figure 6.8) and the Pielou regularity index (evenness) (not shown) were calculated by using qPCR results. The control-SBR had similar diversity and evenness during the whole study period. In the case of modified-SBR, it had more diversity and evenness than control-SBR, except at 50% real wastewater period as explained earlier.

Discussion

Bacteria Communities Analysis

As expected, the microbial community structure changed when the influent characteristics changed in both SBRs. The concentration of rbCOD and ammonia in influent usually has a strong influence on microbial community changes and has been shown in lab-scale and full-scale activated sludge systems (Wang et al., 2014; Valentin-Vargas et al., 2012). *Zoogloea*, most of them were also found to be core genera and shred

by multiple activated sludge systems from WWTPs (Ju et al., 2014; Ye et al., 2013). Members of *Zoogloea* have long been considered as the typical activated sludge bacteria responsible for the formation of activated sludge flocculation and the improvement of the purification process (Dugan et al., 1992; Wang et al., 2012). As previous studied (Mangrum, 1998; Wanner, 1994), *Zoogloea* population was observed to have decreased due to the decreased F/M ratio, where foods are scarce. On the other hand, *Dechloromonas* is a genus capable of reducing perchlorate and also frequently reported as DNPAO in wastewater treatment plants (Zhang et al., 2012; Kong et al., 2007). Many environmental factors have been shown to affect the percentage of *Dechloromonas* in a bioreactor rather than rbCOD and ammonia, including trace elements, salt concentration, and presence of other electron acceptors (Coates and Achenbach, 2006).

The TCOD (includes soluble and particle COD) was related to the microbial distribution (Liu et al., 2007; Wang et al., 2014; Wen et al., 2015). For a given system, the carbon source potentially has a stronger impact on denitrifying community structure than other factors (Lu et al., 2014). In this study, genera *Azoarcus, Thauera,* and *Acidovorax* were found to be the major denitrifiers. These three genera were usually found in industrial/municipal wastewater treatment plants (Lu et al., 2014). When the influent was synthetic wastewater, *Acidovorax* was more abundant than other two denitrifiers. The carbon source for *Acidovorax* can be ethanol, acetate, and polyhydroxyalkanoates (PHAs) (Heylen et al., 2008). When the portion of wastewater in the influent changed (i.e., TCOD increased), other genera increased resulting in more diverse denitrifiers in both SBRs. *Azoarcus* can use methanol, ethanol, acetate, and aromatic compounds as the carbon source for denitrification (Mechichi et al., 2002),

while *Thauera* uses acetate and aromatic compounds (Jiang et al., 2012). *Rhodocyclus* were the core genera in many wastewater treatment plants, which were reported to be responsible for performing anoxygenic photosynthesis under anoxic conditions with a variety of organic compounds as carbon and electron sources (Loy et al., 2005). Consistent with the previous study (Loy et al., 2005), the portion of *Rhodocyclus* increased in both reactors when the carbon sources increased. The genera *Aeromonas* (capable of phosphorus accumulation) and *Metallibacterium* (Fe(III) reducer) were all heterotroph and found to be increased as the organic resources increased (Sidat et al., 1999).

There were two genera, which were *Thiobacillus* and *Nitrosomonas*, in this cluster that was not explainable by the changed of TCOD. *Thiobacillus* is an autotrophic facultative anaerobic bacterium known for its ability to couple denitrification to inorganic sulfur-compound oxidation (Beller et al., 2006). Therefore, it is widely used for denitrification processes of groundwater and industrial wastewater treatment. In addition, the genus *Thiobacillus* is responsible for thiocyanate biodegradation, which is ubiquitous in thiocyanate containing wastewater treatment systems (Felföldi et al., 2010; Zhu et al., 2013). The high concentrations of nitrate nitrogen together with thiocyanate in WWTPs create an ideal niche for *Thiobacillus* sp. The *Nitrosomonas* can be affected by pH, temperature, DO, and some micronutrients (Rostron et al., 2001; Loveless et al., 1968). As a result, the changes of these two specific genera might be because of the 6.3% of community variance. This result could not be explained by the components mentioned above. It may be contributed to other unmonitored wastewater and operational factors that play an influential role in shaping the bacterial community structures.

The modified system that was found could reduce the sludge on the mainstream reactor (modified-SBR) as was mentioned in Chapter 3. This system incorporated with an anaerobic sidestream reactor of the activated sludge yielded an interesting observation that extremely minimized sludge wasting (thus, substantially long SRT) and can be achieved without causing detrimental effects on sludge settling and effluent quality (Goel and Noguera, 2006; Novak et al., 2007; Sun et al., 2010; Chon et al., 2011a,b). To our best knowledge this is the first study to reveal the composition of bacteria that constitute microbial complex in this unique sludge reduction wastewater treatment process by using next generation sequencing. With the longer SRT, the modified-SBR enriched more filamentous bacteria and slow growing bacteria. The unclassified *Cytophagales* bacterium was under the *Cytophagales* order. This order (*Cytophagales*) is mostly filamentous bacteria, which has an ability to degrade cellulose substance and synthesis extracellular polysaccharide (Reichenbach, 2006). *Nitrospira* (NOB), *Mesorhizobium* (nitrogen fixation bacteria), and *Candidatus* Accumulibacter (PAO) are relatively slow growing bacteria. Those could be the reasons why modified-SBR can reduce sludge compared with control-SBR.

Furthermore, the portion of *Niabella* (biopolymer degrader) and *Terrimonas* (nitrate reducer under the aerobic condition (Xie and Yokota, 2006)) in modified-SBR also decreased when the SRT decreased. Our study showed that overall bacterial profile in control-SBR and modified-SBR was different when the reactors were fed with synthetic wastewater, then but fairly similar when the influent was changed to real wastewater. The organics from real wastewater shaped the microbial community than the SRT (even the SRT was 80-days in stage III). So the organics, especially the particle

organics would be the major reason why the sludge reduction in the mainstream reactor decreased with real primary effluent compared with the synthetic feed.

AOBs and NOBs Communities

From the results from TRFLP and Illumina Miseq, only *Nitrosomonas* was detected as the AOB in all of the samples as shown in previous studies (Siripong et al., 2007; Bai et al., 2012). *Nitrosomonas* is resistant to the changing environment and has a relatively high growth rate compared to *Nitrosospira*, so *Nitrosomonas* was more dominant AOB in the activated sludge process (Ma et al., 2015). Based on TRFLP profiles (Figure 6.4), it appeared that the modified-SBR has greater diversity of ammonia oxidizing bacteria than in the control-SBR, except during stage III. *N. oligotropha* has been reported as the dominant AOB in chloraminated drinking water systems (Purkhold et al., 2000) and wastewater treatment plants (Wahman et al., 2011). *N. communis* was also found in the wastewater treatment plants (Tokuyama et al., 2004). *N. cryotolerans* and *N. marina* are found in extreme low temperatures (Karkman et al., 2011) and in saline or marine environments (Ward et al., 2000), respectively. In the end, both reactors only contained *N. europaea/eutropha* related AOBs.

The Illumine Miseq results were similar to the results of TRFLP, but they had more species were involved. Nitrosomonas Stercoris affiliated with *N. eutropha* (96% sequence similarity), usually was found in high ammonia concentration. *N. ureae* can use urea as ammonia source (Koops et al., 1991). Those two species appeared after the influent changed to 50% of real primary effluent. Moreover, Illumine Miseq results also showed the percentage of AOBs in modified-SBR was higher. Overall, both Illumina Miseq and TRFLP results showed that modified-SBR contained more diverse AOBs. The

reason of this difference in both SBRs could be the sidestream reactor attached to the modified-SBR, which can provide the extreme conditions. This condition was able to select and store some organisms. However, when the modified-SBR fed with the real primary effluent continuously, the previous microorganisms had to become accustom to the new environment.

For the TRFLP results of *Nitrobactor*-related NOBs, NIT3r primer has one mismatch to several *Rhodopseudomonas* and *Bradyrhizobium* bacteria, which are ubiquitous and may have contributed to the other peaks (Siripong and Rittmann, 2007). The *Nitrospira* communities in both SBRs were similarly based on TRFLP profiles. From the Illumina Miseq results, a much lower abundance of *Nitrobacter* was found as compared with *Nitrospira* in present study, also suggesting that *Nitrospira* is the major NOB in both SBRs. Higher percentage of *Nitrospira* was found in the modified-SBR than in the control-SBR. Periodic nitrite accumulation in the control-SBR was recorded and became lower than $0.5 mgNL^{-1}$ when the reactor was running at steady state with real wastewater. It contained sufficient alkalinity, while no nitrite accumulation was observed in the modified-SBR. Because of either less NOBs in the control-SBR or more diversity of AOBs in the modified-SBR, it is inconclusive at this stage as to why occasional nitrite accumulation was recorded in the control-SBR.

PAO and DNPAO Communities

DNPAOs have metabolic characteristics similar to those of PAOs, based on the metabolic transformations responsible for enhanced biological phosphorus removal (EBPR) (Tsuneda et al., 2006; Ahn et al., 2002). In a similar manner as PAOs, DNPAOs also take up external carbon substrates and store it as PHAs in the cell under anaerobic

conditions. However, they can utilize nitrite or nitrate instead of oxygen as an electron acceptor to remove phosphorus without any extracellar carbon substrates under anoxic conditions. So in both SBRs, anoxic phosphate uptake and denitrification can be simultaneously performed by DNPAOs under anoxic conditions without any carbon substrate, because nitrification during aerobic conditions provides an electron acceptor for anoxic phosphate uptake. Most DNPAOs are able to utilize oxygen as well as nitrate (Kim et al., 2013). Thus, even if DNPAOs are dominant, aeration for nitrification allows phosphate uptake using O_2, which results in limited phosphate uptake using nitrate under subsequent anoxic conditions. Previous studies (Lv et al., 2014; Kong et al., 2007) showed that some *Dechloromonas spp.* could take up short chain fatty acids and accumulate PHA and polyP, thus exhibiting PAO phenotype. *Dechloromonas*-related bacteria had been detected in several EBPR processes operated under partially anoxic conditions (Kong et al., 2007; Tsuneda et al., 2006).

Miyake and Morgenroth (2005) and Pijuan et al., (2009) evaluated the effects of starving conditions on PAOs and have shown that PAOs can use their intracellular polymers, glycogen and/or polyphosphates as their energy source during period of starvation. However, very few studies focused on evaluating the effect of long SRT or starvation conditions on PAOs ecology. In this study, PAOs belonging to clades IA, IIA, IIC, and IID were present in both SBRs. Using *ppk1* as a biomarker, a number of different clades (IA, IC, IIA, and IID) have been found in laboratory-scale SBRs in several previous studies (He et al, 2010; Slater et al., 2010; Kim et al., 2010; Peterson et al., 2008; Wilmes et al., 2008). Furthermore, clades IIA, IIC, IID were identified as dominant clades in the wastewater treatment plant. Also, it has been shown that the PAOs

belonging to clade IA were able to take up phosphorus using nitrate as the final electron acceptor, and had denitrifying P uptake properties (Kim et al., 2013; Flowers et al., 2009). Zeng et al. (2013) investigated that PAOs in clade IID can use nitrite as an electron acceptor for denitrifying P removal. As mentioned before, the denitrification was observed during the last anoxic phase of each cycle and we attributed the reason to the presence of DNPAO. PAOs belonging to clades IA and IID, which have been shown to have denitrifying capabilities, were present in both SBRs. Hence, it was not surprising that denitrification in the absence of soluble rbCOD was observed during the last anoxic phase in both SBRs.

Summary

The Illumina Miseq analysis revealed that the diversity of microbial communities in both SBRs with synthetic wastewater as influent were lower than those fed with real primary effluent. The CCA results illustrated that influent concentration of rbCOD, TCOD, NH_4^+-N, and SRT were correlated most strongly to the variance of bacterial communities. The sludge minimizing bioreactor enriched more slow growing bacteria and filamentous bacteria than control-SBR when the influent was synthetic wastewater and 25% of real primary effluent. The microbial communities in both SBRs were shaped by the TCOD, and became similar when both SBRs were fed with 100% real wastewater. The changes of microbial communities in the sludge minimizing bioreactor would be one of the reasons which contributed to less sludge minimization with real wastewater than with synthetic wastewater. The sludge minimizing bioreactor showed more diverse AOBs ecology than the control bioreactor during the time when real primary effluent was the influent. The diversity of NOBs and PAOs in the modified-SBR was similar as that in

control-SBR. The present of *Dechloromonas*-related PAOs, which were DNPAOs, were discovered in both SBRs. These results will significantly contribute to pilot and full-scale applications of simultaneous sludge reduction and nutrient removal with established design practices.

Table 6.1: Bacteria diversity indices from control and modified-SBR

	% S/% W(V/V)	No. of sequences	OTU	Shannon index	Evenness
Control-SBR	100/0	49286	493	2.74	0.5
Modified-SBR		47569	314	2.5	0.5
Control-SBR	25/75	49584	316	2.22	0.43
Modified-SBR		44832	282	2.17	0.43
Control-SBR	50/50	37603	422	3.15	0.6
Modified-SBR		34699	746	3.48	0.62
Control-SBR	0/100	41385	839	3.63	0.64
Modified-SBR		72392	1107	3.94	0.67

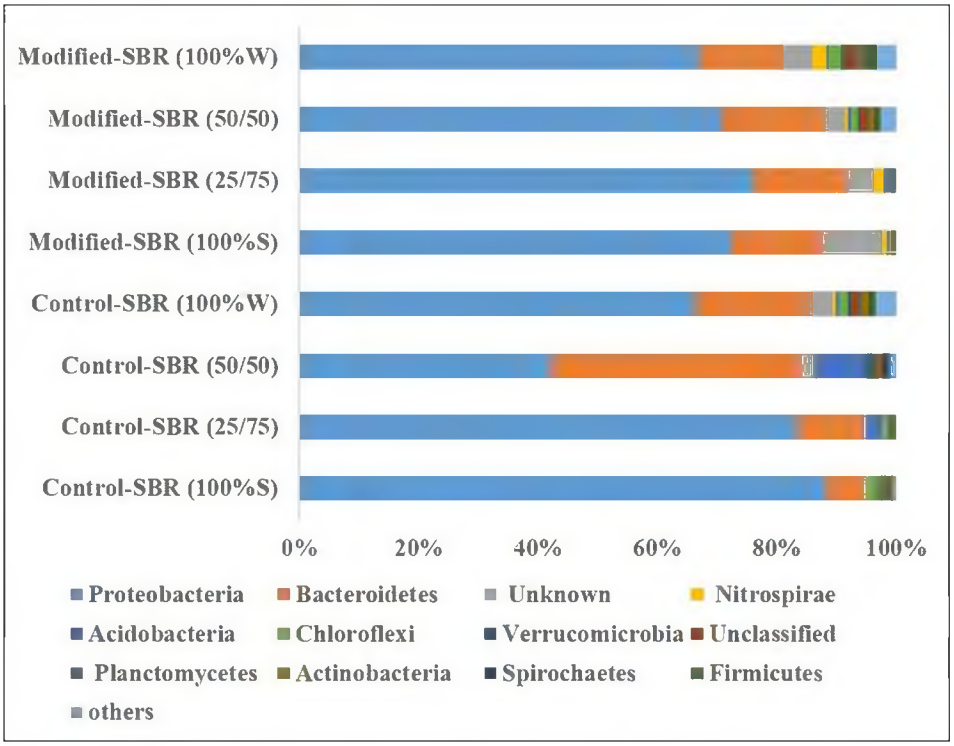

Figure 6.1: Relative abundance of total bacteria grouped by phyla in all samples

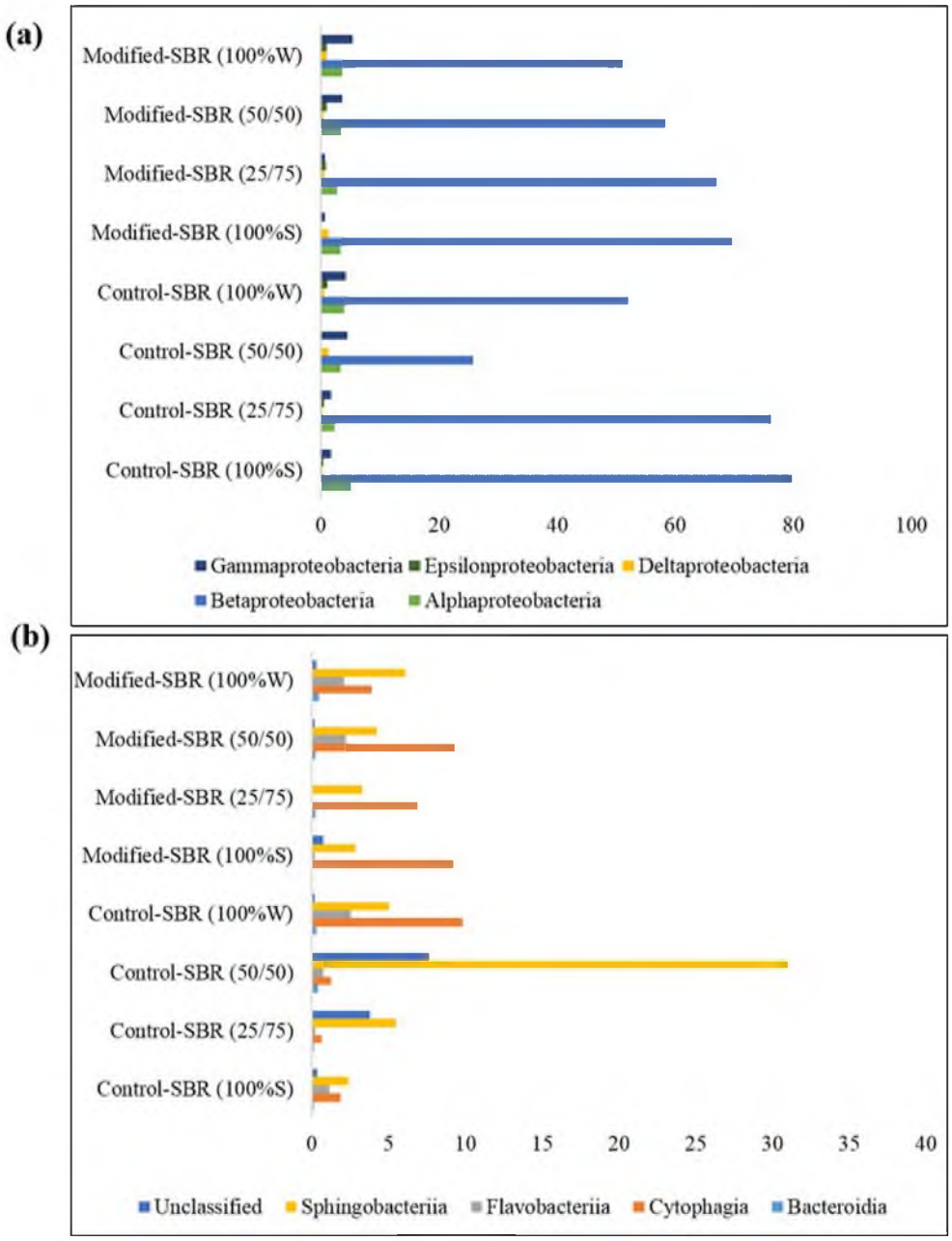

Figure 6.2: Relative abundance of phylum Proteobacteria (a) and Bacteroidetes (b) in all of the samples.

Table 6.2: Percentage of the major genera in each sample (M means the numbers of samples with the genus percentage above 1%). Sample name of "CR" represents control-SBR and "MR" represents modified-SBR, the number in the sample number represents the portion of real primary effluent.

Phylum	Class	Genus	Number	CR0	CR25	CR50	CR100	MR0	MR25	MR50	MR100	M
Acidobacteria	Acidobacteria	Unclassified	1	0.29	2.65	8.77	0.45	0.75	0.05	0.40	0.28	2
Bacteroidetes	Cytophagia	Unclassified	2	1.86	0.55	0.95	1.58	9.09	7.11	9.10	3.10	6
	Flavobacteria	Flavobacterium	3	0.81	0.16	0.52	1.29	0.03	0.02	1.02	0.70	2
	Sphingobacteria	Niabella	4	0.04	0.28	0.23	0.02	0.59	1.00	0.01	0.02	1
		Terrimonas	5	0.04	0.28	0.47	0.10	1.65	1.80	0.10	0.05	2
		Unclassified	6	2.27	4.77	29.82	5.19	1.50	2.41	4.39	5.75	8
	Unclassified	Unclassified	7	0.39	4.43	8.48	0.43	2.83	8.86	0.35	0.91	4
Chloroflexi	Chloroflexia	Oscillochloris	8	1.24	0.87	0.00	0.01	0.05	0.08	0.02	0.00	1
Nitrospirae	Nitrospira	Nitrospira	9	0.02	0.01	0.25	0.52	1.58	0.73	0.41	2.25	2
Proteobacteria	Alphaproteobacteria	Mesorhizobium	10	0.09	0.10	0.12	0.16	1.86	2.27	0.15	0.25	2
		Sphingomonas	11	0.24	0.10	0.18	1.61	0.32	0.30	1.57	0.95	2
		Unclassified	12	0.03	0.01	0.10	0.10	1.34	0.75	0.03	0.11	1
	Betaproteobacteria	Acidovorax	13	3.34	1.17	4.20	2.53	2.09	1.26	2.73	3.18	8
		Piscinibacter	14	0.02	0.14	1.15	0.04	0.56	0.67	0.05	0.04	1
		Candidatus Nitrotoga	15	0.00	0.00	1.20	0.00	0.01	0.00	0.01	0.01	1
		Thiomonas	16	7.74	0.00	0.00	6.44	1.22	0.00	0.00	0.70	3
		Thiobacillus	17	0.03	0.02	0.28	1.22	1.27	1.44	1.29	5.65	5
		Nitrosomonas	18	0.49	0.13	0.51	1.60	1.49	0.51	1.58	4.53	4
		Azoarcus	19	0.34	0.08	0.07	2.13	0.22	0.05	3.15	1.76	3
		Dechloromonas	20	42.07	22.00	1.35	5.74	2.16	3.23	5.91	3.41	8
		Rhodocyclus	21	5.16	0.56	2.74	11.64	1.92	1.83	12.95	5.40	7
		Thauera	22	0.63	1.72	2.14	2.19	1.87	0.23	2.21	3.83	6
		Zoogloea	23	30.21	24.55	0.58	2.24	5.23	3.72	2.37	2.17	7
		Candidatus Accumulibacter	24	7.14	4.81	5.29	12.28	47.15	52.14	14.88	10.65	8
		Unclassified	25	0.04	0.03	2.90	1.41	1.23	0.05	1.22	5.98	5
	Gammaproteobacteria	Aeromonas	26	0.09	0.08	0.08	0.39	0.05	0.12	0.18	1.33	1
		Metallibacterium	27	0.59	0.00	0.00	1.33	0.00	0.00	1.38	0.05	2
	Unclassified	Unclassified	28	0.29	1.71	5.78	0.38	1.01	0.38	0.32	0.85	3
Spirochaetes	Spirochaetia	Turneriella	29	0.00	0.00	1.19	0.01	0.03	0.00	0.01	0.02	1
Unclassified Bacteria			30	0.63	0.23	1.08	2.38	0.39	0.53	2.01	3.66	4
Others			31	13.95	8.47	19.37	26.57	11.72	8.46	22.97	32.43	8

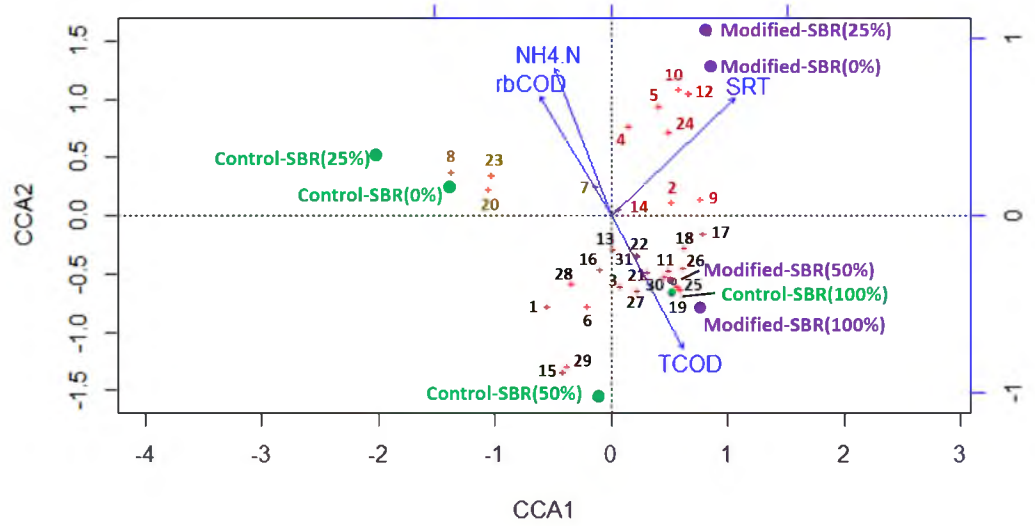

Figure 6.3: Canonical correspondence analysis (CCA) of Miseq data and environmental variables in the eight samples from both SBRs. The number represent the genera showed in Table 6.2 electropherograms obtained on the DNA samples of the mixed liquor in the control and the modified reactor.

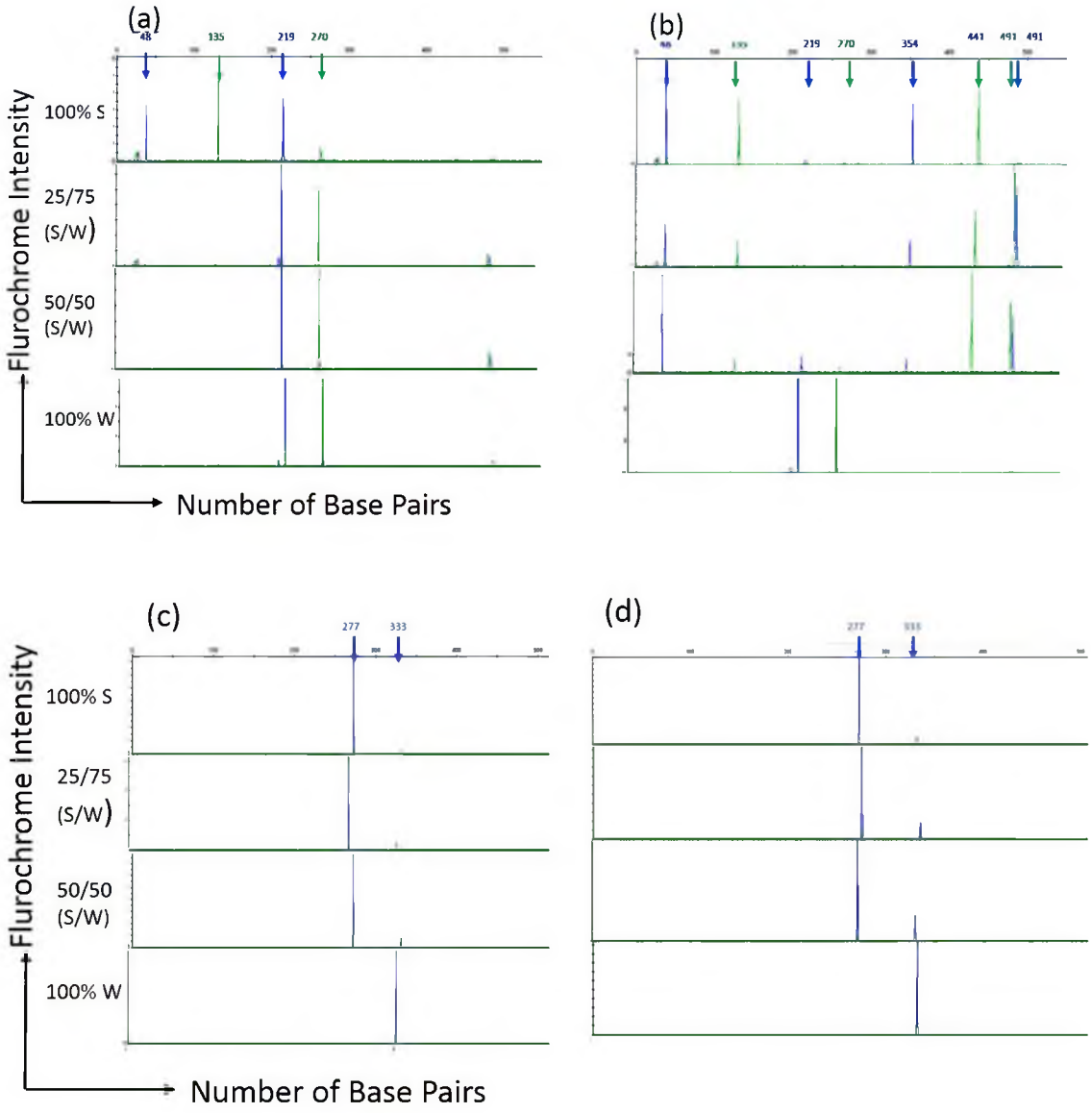

Figure 6.4: Chromatograms representing TF (Terminal Fragments) analysis of the amoA genes and Nitrospira obtained from control-SBR (a and c) and modified-SBR (b and d). The x-axes indicate5'-terminal fragment size in base pairs and the y-axes shows fluorescent intensity.

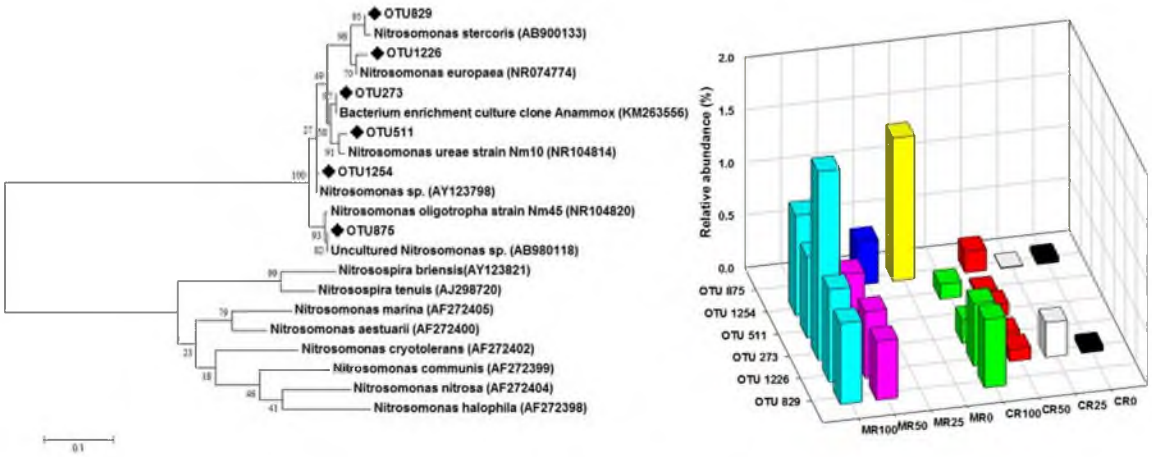

Figure 6.5: Maximum likelihood tree (left hand side) generated from an alignment of Nitrosomonas-related OTUs from both reactors with respect to representative Nitrosomonas genus sequences obtained from other studies (the bar represents 0.1 estimated changes per nucleotide); relative abundance of each OTU per sample (right hand side).

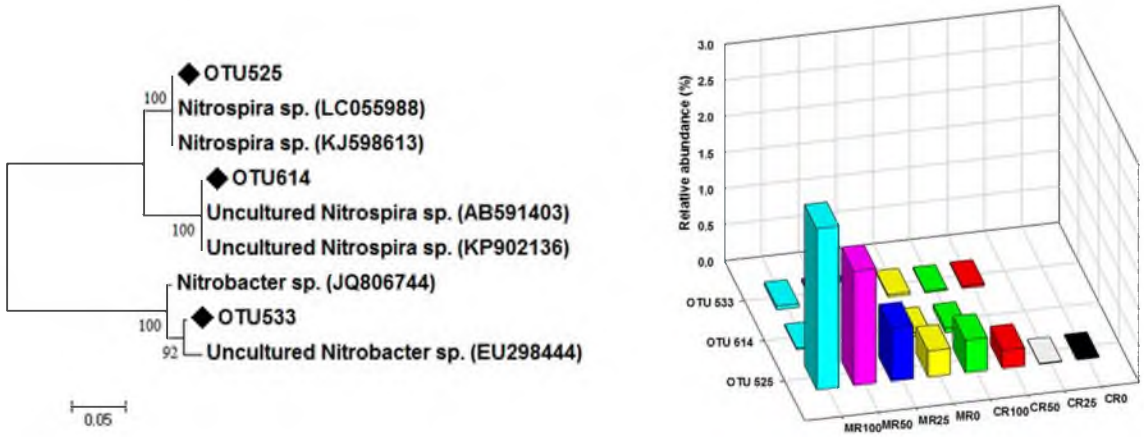

Figure 6.6: Maximum likelihood tree (left hand side) generated from an alignment of Nitrospira and Nirobacter-related OTUs from both reactors with respect to representative Nitrospira and Nirobacter sequence obtain from other studies (the bar represents 0.05 estimated changes per nucleotide); relative abundance of each OTU per sample (right hand side).

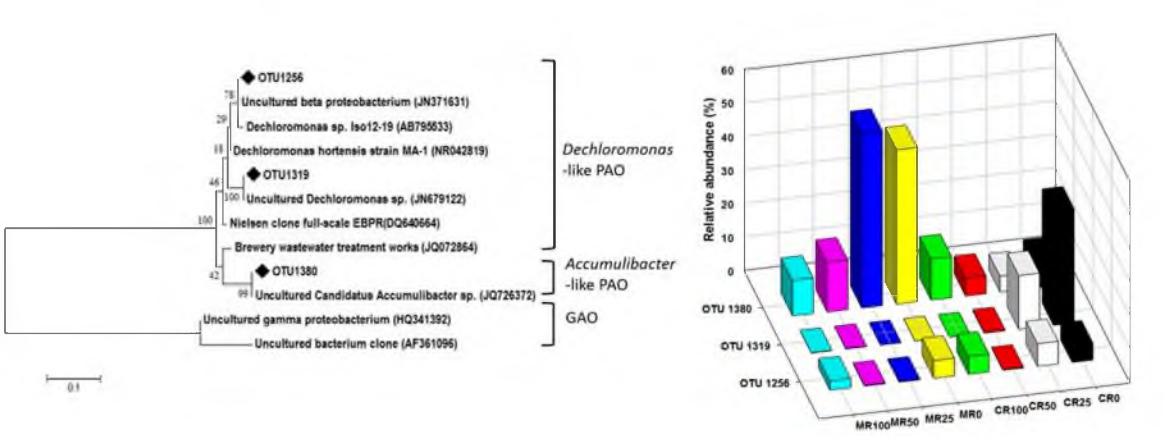

Figure 6.7: Maximum likelihood tree (left hand side) generated from an alignment of OTUs assigned to Candidatus Accumulibacter and Dechloromonas related PAOs from both reactors with respect to representative *Candidatus* Accumulibacter and Dechloromonas sequence obtain from other studies (the bar represents 0.1 estimated changes per nucleotide); relative abundance of each OTU per sample (right hand side).

Table 6.3: Distribution representing the relative abundance generated based on qPCR quantification of ppk1 clades in the both SBRs during this study.

	% S/% W(V/V)	IA	IIA	IIC	IID
Control-SBR	100/0	0.6%	1.7%	90.4%	7.3%
Modified-SBR		3.4%	2.9%	87.3%	6.4%
Control-SBR	25/75	1.2%	0.8%	89.8%	8.2%
Modified-SBR		0.3%	1.4%	88.5%	9.7%
Control-SBR	50/50	0.4%	0.7%	85.0%	13.9%
Modified-SBR		0.2%	5.4%	92.9%	1.5%
Control-SBR	0/100	3.1%	3.3%	89.4%	4.2%
Modified-SBR		4.0%	2.5%	82.3%	11.2%

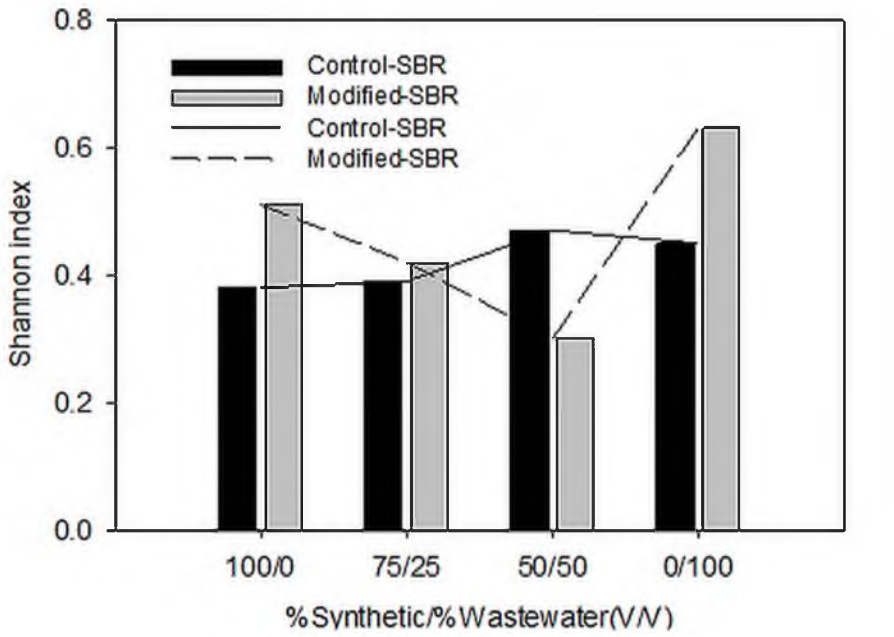

Figure 6.8: Shannon index of the "Candidatus Accumulibacter" lineage in both SBRs

CONCLUSIONS

This study has provided an analysis of the feasibility of coupling the sludge minimization process with nutrients removal. From this research, several conclusions can be derived.

The lab-scale control-SBR, which was maintained for 10-days of SRT, and the modified-SBR when operated at a sufficiently high SRT, performed stable nutrients removal as both SBRs were fed with synthetic wastewater and real wastewater.

The modified-SBR performed slightly better than the control-SBR on NH_4^+-N and PO_4^{3-}-P removals. The bacterial ecology analysis showed that the modified-SBR contained more diverse AOBs and PAOs, than in the control-SBR.

The overall observed sludge reduction in the modified-SBR, as compared to the sludge yield in the control-SBR, also decreased from 65% to 39% and eventually dropped to 35%. This occurred when the feed was changed from the synthetic to the primary effluent and then to raw wastewater. The loss of slow growing bacteria in the modified-SBR could be one of the reasons the sludge reduction decreased.

The mechanism for the solids loss appears to result from the carbon mass balance, due to the maintenance and endogenous metabolism in the main reactor. Also, it is due to the solubilization of organic matter in the sidestream reactor, which is then degraded when the organic matter is returned back to the main reactor.

Phosphorus mass balance was conducted when the reactors were fed with primary

effluent and raw wastewater, with approximately 18% of the phosphorus unaccounted for. The phosphorus rich supernatant in the sidestream reactor, which attached to the modified-SBR, provided the potential of phosphorus recovery during the period when the raw wastewater was fed to the reactor.

This research provided the greater insight into the simultaneous sludge reduction and nutrients removal in the activated sludge process. This information will assist future research focusing on further reducing sludge and enhancing nutrients removal/recovery in the activated sludge process, it hopes of providing a tool for improving our environment.

APPENDIX

Publications

Huang, P., & Goel, R. (2015). Response of a Sludge Minimizing Lab Scale BNR Reactor When the Operation Is Changed To Real Wastewater. *Water Research*, 81, 301-310.

Bhattacharjee, A. S., **Huang, P.**, Mukherjee, S. T., & Goel, R. (2014). New Connectivity between Carbon and Nitrogen Cycles-Nitrite/Nitrate Coupled Methane Oxidation. *Proceedings of the Water Environment Federation*, *2014*(19), 574-583.

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Huang, P., Christensen. H., Mukherji, S. T., & Goel, R. In Preparation. Feasibility Studies on Lab-Scale Ammonia Oxidizing Archaea/ANAMMOX System for Nitrogen

Removal.

Huang, P., & Goel, R. In Preparation. The Microbial Communities Analysis of Activated Sludge from Lab-Scale Simultaneous Nutrients Removal and Sludge Minimization Reactor.

Hogsett, M., **Huang, P.,** & Goel, R. In Preparation. Sediment Oxygen Demand and Nutrient Fluxes in the Eutrophic Utah Lake.

Conference Presentations

Pei Huang, Sachiyo Mukherji, Ramesh Goel. ACS National Meeting and Exposition, Boston, MA, U.S. Aug 2015. Coupling chemical and biological processes for nutrient recovery and removal for better source separated urine management.

Ananda Shankar Bhattacharjee, **Pei Huang**, Ramesh Goel. ACS National Meeting and Exposition, Boston, MA, U.S. Aug 2015. Use of dissolved methane gas for denitrification-process kinetics and microbiology.

Pei Huang, Sachiyo Mukherji, Ramesh Goel. WEF Residuals and Biosolids Conference, Washington DC, U.S. June 2015. Can we minimize biosolids production in activated sludge systems by process manipulations – process sustainability and nutrients removal.

Pei Huang, Sachiyo Mukherji, Ramesh Goel. WEAU (Water Environment Association of Utah), Saint George, UT, U.S. April 2015. Fate of estrogens during biosolids treatment.

Pei Huang, Sachiyo Mukherji, Ramesh Goel. IWA (International Water Association) Sustainable Wastewater Treatment and Resource Recovery Conference, Kathmandu, Nepal. Oct 2014. Fate of estrogens and illicit drugs during urine separation and treatment.

Ananda Shankar Bhattacharjee, **Pei Huang**, Sachiyo Mukherji, Ramesh Goel. WEFTEC (Water Environment Federation's annual Technical Exhibition and Conference), New Orleans, LA, U.S. Sept 2014. New connectivity between carbon and nitrogen cycles – nitrite/nitrate coupled methane oxidation.

Pei Huang, Ramesh Goel. WEFTEC (Water Environment Federation's annual Technical Exhibition and Conference), New Orleans, LA, U.S. Sept 2014. Phosphorus recovery followed by deammonification of urine for nutrient management.

Pei Huang, Sachiyo Mukherji, Ramesh Goel. WEAU (Water Environment Association of Utah), Saint George, UT, U.S. May 2014. Fate of estrogens during urine separation and management.

Amir Motlagh, **Pei Huang**, Ksheeraja Yakkala. WEFTEC, National Design Competition,

Chicago, IL, U.S. Oct 2013. South Davis Sewer District (SDSD) feasibility study for methane production enhancement using fats oil and grease (FOG) anaerobic digestion augmentation.

Mitch Hogsett, **Pei Huang**, Ramesh Goel. AEESP Colorado School of Mines, Golden, Colorado, U.S. July 2013. Water Sustainability through Surface Water Quality Sediment-Water interactions in an urbanized stream.

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